Use of Empirically Optimized Perturbations for Separating and Characterizing Pyloric Neurons

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This dissertation titled

Use of Empirically Optimized Perturbations for Separating and Characterizing Pyloric Neurons

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ABSTRACT

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Conductance based neuron models are commonly used to simulate the activity of real neurons. A challenge in constructing these models is that it is not currently possible to measure all parameters from a single neuron, so measurements are typically made in multiple neurons of a given type. However, neurons can produce the same activity with different parameters, and models constructed with measurements across neurons can produce incorrect behavior. A possible alternative method is fitting model parameters with recordings taken from a single neuron stimulated with a perturbation which elicits information rich responses. Investigations of the parameter space of a pyloric neuron model reveal widespread regions of neurons of nearly identical activity, so perturbation is necessary to constrain parameters. Optimized voltage clamp perturbations are designed using a genetic algorithm to elicit distinctive responses from the voltage-dependent conductances. These perturbations demonstrate superior performance in separating model neurons which behave similarly in free-running conditions, causing differences in their responses to reflect differences in parameters, and facilitating gradient descent based parameter fitting methods. When used in real neurons, they cause pyloric neurons of different anatomical types to produce characteristic responses which allow automatic classification that recapitulates their anatomical types. However, parameter fitting of a pyloric model to a real neuron’s responses is likely to require multiple compartments and multiple perturbations due to the anatomical complexity of these neurons.
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CHAPTER 1: INTRODUCTION

Overview of Research

An ongoing goal in neuroscience research is measuring the parameters of voltage-dependent conductances in single neurons. With current methods, a conductance of interest is isolated by chemical blockade, and electrophysiological protocols are used to measure the response of the conductance. These data are then used to fit the parameters of equations describing the conductance. A problem with this technique is that not all of the conductances can be measured in the same neuron, as blockade of unwanted conductances is often irreversible. Thus, measurements must be made from many neurons. However, both neuron models and real neurons can produce similar responses despite having different conductance parameters, so the parameters measured across many different neurons of the same type may not produce a functional model (Chapter 2 - Introduction).

A promising alternative method is to make measurements of all conductances at once, by perturbing the neuron in such a way to elicit an information-rich response. An appropriate model could then be fit to this response by changing the model’s parameters until its response matches the recording from the live neuron. Given the large number of parameters, and the time required to simulate neural activity, this would be done using a heuristic method such as genetic algorithm or simulated annealing. This was attempted in pyloric neurons from the stomatogastric ganglion of the spiny lobster, Panulirus interruptus, using an evolution strategy to adjust the parameters of a pyloric neuron model perturbed with white noise (Hobbs and Hooper, 2008). In these experiments, a model neuron with a fixed set of parameter values was stimulated with white noise, and then an evolution strategy was used to discover these parameter values starting from
random values. When the same technique was applied to try to fit a neuron model to real data recorded from pyloric neurons, however, it was not successful.

This body of work comprises five manuscripts which explore why this was unsuccessful, and consider what steps must be taken to achieve this goal. A single-compartment model of pyloric neurons is used throughout (Prinz et al., 2003), though, limitations of this model are noted and discussed (Chapter 7). At each stage of research, different questions are considered. These include:

1. Can models with different parameter values yield similar activity in this model? If so, how extensive is this problem? If many different parameter values can yield models which behave identically, then the spontaneous activity of model neurons cannot be used to measure parameter values, and instead, appropriate perturbations should be used. This is considered in Chapter 2.

2. What perturbations should be used in model neurons? Is it possible to design a perturbation empirically for maximum effect? One consideration, referred to herein as separation, is whether a perturbation is good for making neurons with similar parameter values respond more similarly to one another, and neurons with different parameter values respond more differently from one another. The second consideration, referred to as pathfinding, is whether the perturbation is compatible with the use of a heuristic algorithm. This is considered in Chapter 3.

3. Do the perturbations identified in Chapter 3 have any value in real neurons? Can they actually make neurons with different characteristics behave differently, but in predictable ways? Pyloric neurons are classified into types based upon their muscle innervations, with different neuron types active at different phases of the pyloric rhythm. If different types are reflected as differences in parameter values, and the perturbations good at
separation in model neurons are also good at separation in real neurons, they should elicit responses which differ by neuron type. If however these perturbations are unable to make different neuron types behave differently, it is unlikely that future research in real neurons, such as attempting the equivalent of pathfinding using heuristic methods, will be successful. This is considered in Chapter 4.

4. Are these perturbations likely to penetrate the neuron well enough to extract enough information for the genetic algorithm to fit parameter values? And how much complexity is required to model a real neuron? In previous stages of research, a single compartment model was used. However, real pyloric neurons are anatomically complex and may require more sophisticated modeling. These questions are considered in Chapter 6 by constructing detailed anatomical models of pyloric neurons from confocal images. A related concern was whether these anatomical models are sufficient; this is addressed with membrane capacitance measurements made in Chapter 5.

5. What recommendations can be made regarding perturbation and appropriate neuron models in future research towards the goal of measuring conductance parameters with a heuristic algorithm? This is briefly considered in Chapter 7.

The manuscript of Chapter 5 has been submitted for publication, and is currently undergoing revision for resubmission. The remaining manuscripts (2, 3, 4, and 6) will be submitted subsequent to completion of the dissertation. Manuscripts for the algorithms described in Appendix 1 will be submitted to methods journals as appropriate.
Terminology

To avoid ambiguity, the phrase neuron model will be used to refer to a well-defined mathematical model of a neuron with free parameters, whereas model neuron will refer to a neuron model whose parameters have been assigned specific values. Note that the same neuron model is used throughout this research. Unless otherwise specified, a parameter refers to a conductance parameter. Where clear from context, and especially in figures, these conductance parameters are commonly referred to by their subscripts, e.g., $g_{Kd}$ is written as Kd. In Chapter 3, where the parameters of a waveform being adjusted by a genetic algorithm are discussed, the phrases conductance parameter and waveform parameter are used consistently to avoid ambiguity.

Pyloric neurons are identified into types according to the muscles they innervate. These types are commonly referred to by abbreviations, used throughout. They include:

- AB (Anterior Buster), a pacemaker neuron
- PY (Pyloric)
- LP (Lateral Pyloric)
- PD (Pyloric Dilator)
- VD (Ventricular Dilator)
- IC (Inferior Cardiac)
CHAPTER 2: THE REGION OF ISOCRONOUS PYLORIC NEURON MODELS IS A CURVED HYPERSHEET

Author Contributions

The research in this chapter was performed in an attempt to understand the nature and limitations of the pyloric neuron model used in ongoing work in the S. L. H. lab. W. E. W. originated the idea to characterize the parameter space of this model under free-running conditions in low and high resolution, and developed the techniques necessary to perform this task. W. E. W. wrote all software and performed all experiments in this section. S. L. H contributed to research design and interpretation of results. Upon review of the results, S. L. H. suggested expanding this research with the goal of submitting a manuscript for publication. W. E. W. prepared all figures and wrote the manuscript. S. L. H. and all dissertation committee members assisted in editing the manuscript and figures, with W. E. W. revising making revisions according to their feedback.

Abstract

Conductance-based neuron models are frequently used in neuroscience research to reproduce neuronal activity. A known concern with both conductance models and real neurons is that many different combinations of parameter values can yield neurons which produce identical behavior under free-running conditions. Using a single-compartment model of pyloric neurons in the lobster, *Panulirus interruptus*, and a bursting pacemaker neuron as a reference, the nature and extent of the regions of parameter space yielding models behaving similarly to the reference were characterized. Similarity was assessed using two different distance metrics, waveform distance and phase plane distance. Regardless of distance metric, these regions were found to range
widely throughout parameter space. The region of similarity with waveform distance was a thin, highly curved hypersheet of dimension close to 2, extending throughout parameter space.

Introduction

Conductance-based neuron models

Conductance-based neuron models are commonly used to simulate the electrical activity of a neuron. Such models simplify its biophysical complexity to an equivalent circuit of one or more connected electrical compartments. These compartments are isopotential, i.e., having the same membrane potential throughout. While anatomically accurate neuron models may require hundreds or even thousands of compartments, many neuron models are implemented with only a single isopotential compartment. Each electrical compartment contains passive electrical elements representing membrane capacitance and leak conductance, and may contain active conductances representing the activity of ion channels in the real neuron. The flow of currents in this electrical circuit is characterized by a series of ordinary differential equations which represent these components according to Kirchhoff’s and Ohm’s Laws. Voltage clamp is achieved by forcing the membrane voltage of the clamped compartment to conform to the stimulus waveform trace, and current clamp by adding an additional current to the clamped compartment. As these equations can only be solved exactly in the simplest of cases, numerical integrators are used instead to produce approximate solutions of desired accuracy.

Active conductances are often modeled using Hodgkin-Huxley-style ordinary differential equations of the form

\[ I_x = \bar{g}_x m^p h^q (V - E_x) \]
\[ \frac{dm}{dt} = (m_\infty - m)/\tau_m \]
\[ \frac{dh}{dt} = (h_\infty - h)/\tau_h \]
where \( V \) is the time-varying membrane voltage within the compartment, \( \bar{g}_x \) is the maximal conductance, \( E_x \) is the reversal potential of the current, \( m \) and \( h \) are gating variables representing activation and inactivation, and \( p \) and \( q \) are exponents on the activation and inactivation gates.

The steady-state gating functions \( m_\infty \) and \( h_\infty \) are often sigmoidal, i.e.,

\[
\frac{1}{1 + e^{(V - V_h)/s}}
\]

with \( V_h \) being the half-maximal activation voltage and \( s \) the slope. The time constants \( \tau_m \) and \( \tau_h \) may be voltage-independent, or may depend upon voltage as sigmoids or other equations. A single conductance’s equations may have five or more parameters, and a single compartment in a neuron may have many different conductances. A complete model may thus have dozens of parameters per compartment. In practice, however, most of these parameters are given fixed values in a given neuron model; only a few, such as the maximal conductance values (\( \bar{g}_x \)), are free to vary. In multi-compartment models, these free parameters are often assumed to be identical across many compartments in a section.

The set of all possible combinations of values for the free parameters is known as the parameter space of the model, and a point in this parameter space corresponds to a vector of parameter values in much the same way that a point in physical space corresponds to a vector of three-dimensional coordinates. A particular point in a neuron model’s parameter space defines a specific model neuron (herein, neuron model refers to a mathematical model having free parameters, whereas model neuron refers to a model whose free parameters all have values). This model neuron will produce a characteristic waveform trace in response to a given perturbation or under free-running conditions. A different model neuron made by choosing a different point in parameter space may respond similarly under the same conditions, or its response may be quite different. The difference may be great even when the two points are relatively close in parameter space.
space, given the nonlinear nature of the ordinary differential equations. The difference between two waveform traces is quantified using \textit{distance metrics}, functions which associate a scalar distance value with every pair of waveform traces. The commonly used waveform distance metric ($D_w$) for example measures the area difference between two waveform traces, scaled by total sample time. The choice of distance metric is important when trying to assess whether two model neurons are producing similar output, and different distance metrics are sensitive to different features in a waveform, such as phase difference, spike timing, burst frequency, or envelope shape.

\textit{Parameter fitting and the multiple solutions problem}

When neuron models are to be used to reproduce real neuron behavior, the free parameters must be set appropriately from measurements taken from live neurons. Traditional means of estimating neuron conductance parameters involve chemical blockade of the unwanted conductances to isolate the desired conductance, followed by electrophysiological protocols which provide sufficiently rich data to decisively fit the parameter’s equations. The chemical blockade is often irreversible, so multiple experiments are required to measure all conductance parameters. A known concern with this technique is that two neurons which behave identically may have different conductance parameters, and may maintain constant activity by modulating these parameters together (Desai et al., 1999; Golowasch et al., 1999; Haedo and Golowasch, 2006; LeMasson et al., 1993; Li et al., 1996; Liu et al., 1998; MacLean et al., 2003; Marder and Prinz, 2002; Marder and Goaillard, 2006; Mizrahi et al., 2001; Turrigiano et al., 1995). Combining the parameters measured from multiple experiments may thus produce a model whose behavior does not match any of the experimental neurons used (Blumel et al., 2012; Golowasch et al., 2002), so hand-tuning of model parameters is commonly required to reproduce the desired behavior. As a trivial example, consider a quiescent neuron whose resting membrane potential is
due to a balance of two steady-state currents, one inward and one outward. The same potential could be achieved in one neuron with low inward and low outward current, and in another with a correspondingly balanced high inward and high outward current (note however that these neurons would produce different responses to current injection). A composite model made from the measured inward conductance of the first model and the outward conductance of the second would however not have the resting potential of either neuron.

Prior research has established that many neuron models do produce qualitatively or quantitatively similar behavior with different parameters (Achard and De Schutter E., 2006; Goldman et al., 2001; Prinz et al., 2004). In a cerebellar Purkinje neuron model, for example, essentially identical behavior was demonstrated across wide regions of parameter space (Achard and De Schutter E., 2006). With the relatively low resolution with which the parameter space was investigated, these regions appeared as small, locally connected hyperplanes. A frequently used model of pyloric neurons of the lobster *Panulirus interruptus* is the Prinz model (Prinz et al., 2003). This model includes seven voltage-dependent conductances and a leak conductance, and has eight free parameters, the maximal conductance values ($\bar{g}$). Different combinations of parameter values yield model neurons which, in free-running conditions, may be quiescent, spike regularly or irregularly, or burst, as do actual pyloric neurons. A very coarse search of the eight-dimensional parameter space of this neuron model revealed that a bursting model neuron could be constructed with multiple combinations of parameter values, and that these also ranged widely across the parameter space (Prinz et al., 2003). Though qualitatively similar, they differed in characteristics such as frequency, plateau size, and number of spikes per burst (*Figure 1 A-C*). Quantitatively similar model neurons, whose outputs appeared nearly identical and which had low distance by the $D_w$ metric, could also be found by manual search of parameter space (*Figure 1 C-E*; the parameter values of the model neurons are given in *Table 1*).
Figure 1. Pyloric model neurons produce similar outputs despite different parameter values

A-C: Three previously identified pyloric model neurons with qualitatively similar rhythmic bursting waveforms (Prinz et al., 2003). C-E: Quantitatively similar waveforms could also be obtained. For parameter values see Table 1.
Table 1

Example $\bar{g}$ Parameters For Bursting Model Neurons With Similar Output

<table>
<thead>
<tr>
<th></th>
<th>Leak</th>
<th>CaT</th>
<th>CaS</th>
<th>A</th>
<th>KCa</th>
<th>Kd</th>
<th>H</th>
<th>Na</th>
<th>Dw vs C</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0</td>
<td>2.5</td>
<td>6</td>
<td>50</td>
<td>10</td>
<td>125</td>
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<td>5</td>
<td>125</td>
<td>0.01</td>
<td>300</td>
<td>13.5</td>
</tr>
<tr>
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<td>5</td>
<td>4</td>
<td>40</td>
<td>5</td>
<td>125</td>
<td>0.01</td>
<td>200</td>
<td>--</td>
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<td>0.01</td>
<td>165.1</td>
<td>6.9</td>
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<tr>
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<td>5.587</td>
<td>125</td>
<td>0.01</td>
<td>200</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Row heading (A-E) correspond to panels in Figure 1. All $\bar{g}$ values are in mS/cm$^2$. $D_w$ vs C is the waveform distance calculated vs neuron C, with a 60 s runtime.

The extent to which different combinations of parameter values can produce model neurons with similar responses is important for several reasons. A goal in computational neuroscience is inferring the conductance complement of real neurons from models by adjusting the model’s free parameters until it reproduces the real neuron’s activity. The existence of multiple solutions hinders this process, as there is no guarantee that the correct set of parameter values has been found when the model’s behavior conforms to the real neuron’s output. Studies of neuron self-regulation, the means by which a neuron adjusts its parameters dynamically to maintain proper activity, are also affected by these concerns. For example, the topology of the region of similarity could provide insights into how neuron parameters are adjusted together, or how activity is measured by the neuron. Detailed explorations of neuron model parameter spaces, which could offer insight into the extent of this problem, are, however, difficult or impossible due to combinatorial explosion. In a single compartment Prinz model, with 8 free
parameters, exploring only 100 values of each parameter would require $100^8$ (10 billion billion) integrator simulations. Even with advances in computational resources, exhaustive search of parameter spaces to any reasonable resolution is thus impossible except for the most trivial of models.

**Overview of research**

To circumvent the combinatorial explosion problem, we developed techniques for high-resolution exploration of the region of parameter space giving rise to similar model neurons, and applied these techniques to the Prinz neuron model. As a reference, we used an arbitrary but known set of parameter values from prior work (Prinz et al., 2003) which elicits the rhythmic bursting behavior in free-running conditions typical of pyloric neurons. These techniques allowed us to characterize the region of parameter space (called herein the **basin of equivalence**) which corresponds to models which behave similarly to the reference. This was done in three separate stages, explained briefly below, and described in detail in *Materials and Methods*.

1. Two- and three-dimensional “slices” of the larger parameter space were exhaustively raster-scanned and visualized at low resolution, by iterating two or three parameters across a defined range in regular intervals, while holding all other parameters at the reference model values. The output of each model neuron was compared to the reference using a distance metric, to produce a map associating each point in the parameter space slice with a distance from the reference. This map was visualized as a “fitness landscape” to guide further explorations. Fitness landscapes were constructed for all combinations of two of the eight parameters, and for all combinations of three of the eight parameters.

2. The region in each three-dimensional slice of parameter space which produced models similar to the target (i.e., the *basin of equivalence*) was explored in high detail. This was done using a vector-based random walk to find seed points, followed by a flood fill
algorithm which expanded from those points on a high-resolution grid until the boundary of the basin of equivalence was reached. This improved visualizations, and provided topological insight about the shape of the region. Again, this was repeated for all possible three-dimensional slices.

3. The full parameter space was then explored using the same flood fill process, and the same topological analysis was performed, to characterize the region. Based upon preliminary results, the complexity of this task was further reduced by one or two simplifying assumptions. First, the leak conductance was often eliminated. This conductance was not present in the reference model neuron, and its presence in other model neurons attenuated the response waveform in a relatively trivial and predictable way. Second, the sodium conductance was often blocked. This provided several advantages. It decreased computational resources, as the sodium conductance equations are “stiff” and require small integrator time steps. Its removal eliminated sodium spikes which were not effectively measured by the distance metrics used in this research. Finally, future in vivo pyloric neuron experiments were planned using the sodium channel blocker tetrodotoxin (TTX) to isolate neurons, and removing this current from the model yielded results more directly applicable to such work.

Frequency is a basic feature of rhythmic neuronal activity, and is presumably maintained within physiologically appropriate bounds by regulatory activity. Frequency is thus an obvious feature to investigate when comparing model neuron responses. When used with long sample times and an initial phase-locking current pulse, the commonly used waveform distance $D_w$ is especially sensitive to burst frequency, and relatively insensitive to spike timing, spike number, or phase differences. We thus chose $D_w$ as the primary distance metric for these investigations, as a means to identify regions of parameter space which were generally isochronous (i.e., having the same frequency). A second metric with similar insensitivity to spike features and phase, phase
plane distance ($D_P$), which compares the trajectories of the neurons in phase space, was utilized for comparison purposes. These metrics and their response characteristics are explored in further detail in *Materials and Methods*.

A final matter is the issue of how to characterize the shape of higher-dimensional objects, as visualization of spaces in excess of three dimensions is difficult. Various techniques exist to estimate the dimensionality of objects in higher dimensional spaces. We chose the Minkowski-Bouligand (box-counting) method, commonly used for estimating the fractal dimension of objects (Falconer, 2003). This method assigns a fractional dimension to an object by counting the number of $n$-dimensional boxes required to cover the object, and analyzing the increase in count as the boxes are shrunk. This dimension provides insight into the morphology of the object. Linear objects have dimensions near 1.0, planes and thin surfaces near 2.0, and filled volumes such as spheres and cubes are near 3.0. Fractal objects which have complexity at all scales have non-integer dimensions. Coastline edges, for example, have box-counting dimension between 1 and 2; mountain ranges and similar surfaces have dimension between 2 and 3.

**Terminology and Definitions**

Given a neuron model $M$ with $n$ free parameters, the set of all possible combinations of values to these parameters forms an $n$-dimensional *parameter space* $P_M$. A specific point $p$ in parameter space corresponds to a vector of $n$ parameter values, which *generates* the specific model neuron $M(p)$ (again, *neuron model* refers to the mathematical model with free parameters, whereas *model neuron* refers to a model whose parameters have assigned values). Assuming fixed initial conditions, $M(p)$ will produce a unique response waveform trace $r$ for any given stimulus (perturbation) waveform trace $s$, i.e., $M(p,s) = r$. When $s$ is a current clamp waveform, $r$ is the voltage response of the model neuron; when $s$ is a voltage clamp waveform, $r$ is the clamp current response. Given a distance metric $D_X$, two points in parameter space $p_1$ and $p_2$, and a
perturbation \( s, D_X(M(p,s), M(p_{\text{ref}},s)) \geq 0 \). For notational simplicity, this is referred to as \( D_X(p,p_{\text{ref}}) \); the perturbation and model are assumed from context.

Given a model \( M \), a distance metric \( D_X \) by which to compare two response waveforms, a perturbation \( s \), and a reference parameter vector \( p_{\text{ref}} \) in \( P_M \), there exists a mapping from every point \( p \) in \( P_M \) into \( R^+ \) (the positive real numbers), being \( D_X(M(p,s), M(p_{\text{ref}},s)) \). We refer to this mapping as the difference landscape for this model, distance metric, perturbation, and reference:

\[
L(M, D_X, s, p_{\text{ref}}): P_M \rightarrow R^+
\]

This difference landscape is conceptually similar to a fitness landscape, and can be visualized similarly, as an \( n+1 \) dimensional surface, where the first \( n \) dimensions define a point \( p \) in parameter space, and the “height” in the \( n+1 \) dimension is the distance \( D_X(M(p,s), M(p_{\text{ref}},s)) \). Higher altitude on the surface corresponds to greater difference from the reference model’s output. The height is zero where \( p = p_{\text{ref}} \).

Given a model \( M \), a distance metric \( D_X \), a perturbation \( s \), and a reference parameter vector \( p_{\text{ref}} \), for every \( \varepsilon > 0 \), there exists a non-empty set \( B(M, D_X, s, p_{\text{ref}}) \), the basin of equivalence for a given model, distance metric, perturbation, and reference parameter vector, such that

\[
\forall p \in B, D_X(M(p,s), M(p_{\text{ref}},s)) < \varepsilon
\]

In other words, the basin of equivalence is the set of all points in parameter space which generate model neurons whose output is similar to (less than \( \varepsilon \) from, specifically) the reference model’s output, as assessed by the distance metric. This set may contain only the reference point \( p_{\text{ref}} \), or it may be extensive throughout parameter space, if the neuron model’s output is relatively insensitive to changes in parameters, if the distance metric is forgiving, or if \( \varepsilon \) is large. It may be connected or not, topologically simple or complex.
Materials and Methods

Pyloric neuron model

A single compartment implementation of the Prinz model of decapod crustacean pyloric neurons was used exclusively in all model simulations (Prinz et al., 2003). This model consists of seven voltage-dependent currents and a leak current:

\[ i_{\text{ion}} = i_{\text{leak}} + i_{\text{Na}} + i_{c\text{a}r} + i_{c\text{a}s} + i_{A} + i_{K\text{Ca}} + i_{Kd} + i_{H} \]

In current clamp, membrane voltage is calculated during integration from

\[ C \frac{dV_{m}}{dt} = -i_{\text{ion}} + i_{\text{ext}} \]

where \( i_{\text{ext}} \) is the clamp current; note that \( i_{\text{cap}} = C \frac{dV_{m}}{dt} \). In voltage clamp, \( i_{\text{ext}} = -i_{\text{tot}} \) is monitored as the clamp current, and \( V_{m} \) is set to the clamp command voltage. The eight currents are:

\[
\begin{align*}
    i_{\text{leak}} &= A \cdot \bar{g}_{\text{leak}} \cdot (V_{m} - E_{\text{leak}}) \\
    i_{\text{Na}} &= A \cdot \bar{g}_{\text{Na}} \cdot m_{\text{Na}}^{3} \cdot h_{\text{Na}} \cdot (V_{m} - E_{\text{Na}}) \\
    i_{c\text{a}r} &= A \cdot \bar{g}_{c\text{a}r} \cdot m_{c\text{a}r}^{3} \cdot h_{c\text{a}r} \cdot (V_{m} - E_{c\text{a}}) \\
    i_{c\text{a}s} &= A \cdot \bar{g}_{c\text{a}s} \cdot m_{c\text{a}s}^{3} \cdot h_{c\text{a}s} \cdot (V_{m} - E_{c\text{a}}) \\
    i_{A} &= A \cdot \bar{g}_{A} \cdot m_{A}^{3} \cdot h_{A} \cdot (V_{m} - E_{\text{K}}) \\
    i_{K\text{Ca}} &= A \cdot \bar{g}_{K\text{Ca}} \cdot m_{K\text{Ca}}^{4} \cdot (V_{m} - E_{\text{K}}) \\
    i_{Kd} &= A \cdot \bar{g}_{Kd} \cdot m_{Kd}^{4} \cdot (V_{m} - E_{\text{K}}) \\
    i_{H} &= A \cdot \bar{g}_{H} \cdot m_{H}^{4} \cdot (V_{m} - E_{H})
\end{align*}
\]

where \( A = 0.628 \cdot 10^{-3} \text{ cm}^{2} \) is the membrane surface area, \( \bar{g}_{x} \) is the maximal conductance for current \( x \) in mS/cm\(^2\), \( E_{\text{leak}} = -50 \text{ mV} \), \( E_{\text{Na}} = 50 \text{ mV} \), \( E_{\text{K}} = -80 \text{ mV} \), and \( E_{H} = -20 \text{ mV} \). The eight \( \bar{g}_{x} \) maximal conductances are the free parameters of the model. The gating particles \( m \) and \( h \) for each current are calculated by integrating \( \tau_{m} \cdot \frac{dm}{dt} = m_{\infty} - m \) and \( \tau_{h} \cdot \frac{dh}{dt} = h_{\infty} - h \), where the time constants \( (\tau_{m}, \tau_{h}) \) and steady-state activation values \( (m_{\infty}, h_{\infty}) \) for each current are calculated as follows:
Table 2

Steady-State Activation Values and Time Constants for Prinz Model Conductances

\[ m_{\infty}(V) = \frac{1}{1 + e^{(V+25.5)/-5.29}} \]

\[ h_{\infty}(V) = \frac{1}{1 + e^{(V+48.9)/5.18}} \]

\[ \tau_m(V) = 2.64 - \frac{2.52}{1 + e^{(V+120)/-25}} \]

\[ \tau_h(V) = \frac{1.3}{1 + e^{(V+62.9)/-10}} \cdot \left( 1.5 + \frac{1}{e^{(V+34.9)/3.6}} \right) \]

\[ m_{\infty}(V) = \frac{1}{1 + e^{(V+27)/-7.2}} \]

\[ h_{\infty}(V) = \frac{1}{1 + e^{(V+32.1)/5.5}} \]

\[ \tau_m(V) = 43.4 - \frac{42.6}{1 + e^{(V+68.1)/-20.5}} \]

\[ \tau_h(V) = 210 - \frac{179.6}{1 + e^{(V+55)/-16.9}} \]

\[ m_{\infty}(V) = \frac{1}{1 + e^{(V+33)/-8.1}} \]

\[ h_{\infty}(V) = \frac{1}{1 + e^{(V+60)/6.2}} \]

\[ \tau_m(V) = 2.8 + \frac{14}{e^{(V+27)/10} + e^{(V+70)/-13}} \]

\[ \tau_h(V) = 300 + \frac{300}{e^{(V+55)/9} + e^{(V+65)/-16}} \]

\[ m_{\infty}(V) = \frac{1}{1 + e^{(V+27.2)/-8.7}} \]

\[ h_{\infty}(V) = \frac{1}{1 + e^{(V+56.9)/4.9}} \]

\[ \tau_m(V) = 23.2 - \frac{20.8}{1 + e^{(V+32.9)/-15.2}} \]

\[ \tau_h(V) = 77.2 - \frac{58.4}{1 + e^{(V+38.9)/-26.5}} \]

\[ m_{\infty}(V, Ca_i) = \frac{Ca_i}{Ca_i + 3} \cdot \frac{1}{1 + e^{(V+28.3)/-12.6}} \]

\[ \tau_m(V) = 180.6 - \frac{150.2}{1 + e^{(V+46)/-22.7}} \]

\[ \tau_h(V) = \frac{1}{1 + e^{(V+169.7)/-11.6} + e^{(V+26.7)/14.3}} \]

\[ m_{\infty}(V) = \frac{1}{1 + e^{(V+12.3)/-11.8}} \]

\[ \tau_m(V) = 14.4 - \frac{12.8}{1 + e^{(V+28.3)/-19.2}} \]

\[ m_{\infty}(V) = \frac{1}{1 + e^{(V+75)/5.5}} \]

\[ \tau_m(V) = \frac{2}{e^{(V+169.7)/-11.6} + e^{(V+26.7)/14.3}} \]
Calcium reversal potential and flux are calculated as

$$E_{Ca} = 1000 \cdot \frac{RT}{zF} \cdot \log \left( \frac{Ca_o}{Ca_i} \right)$$

$$\tau_{Ca} \cdot \frac{dCa_i}{dt} = -f \cdot i_{Ca} - Ca_i + Ca_\infty$$

where $T = 283.15$ K, $R = 8.315$ J/K/mol, $F = 96485$ C/mol, $z = 2$, $Ca_o = 3000$ µM, $f = 14.96$ µM/nA, $Ca_\infty = 0.05$ µM, and $i_{Ca} = i_{CaT} + i_{CaS}$. This based upon a previously published calcium flux equation (Liu et al., 1998), but with the $f$ factor changed from 0.94 to 14.96. The model was implemented in C using the GNU Scientific Library’s ordinary differential equation integrator functions, with optimizations to improve performance.

**Reference model neuron**

The processes used to characterize parameter space relied upon a reference model neuron for the distance calculations. Model neuron C (Table 1) was selected for this purpose. Under free-running conditions this model neuron produces bursts with multiple spikes interspersed with hyperpolarizing phases (Figure 1 C), typical for rhythmically active pyloric neurons.

**Perturbation**

The goal of this research was to compare model response in free-running conditions. This was achieved by using current clamp with a perturbation waveform $s$ which began with initial 1 s phase-locking current pulse followed by 0 nA for the rest of the perturbation. The phase-locking pulse, used to minimize the effect of phase difference on distance metric calculations, consisted of 100 ms at 0 nA, 100 ms -0.2 nA hyperpolarizing current, and 800 ms 1 nA depolarizing current. The first 5 s of the output waveform trace, during which the models settled into a regular rhythm, were not used in analysis or in distance metric calculations. The total integrator simulation time was 60 s or 174 s, as noted below.
Frequency detection

Periodic response waveform traces for oscillating pyloric neurons typically display two distinct characteristic frequencies (Figure 1). One is the slow-wave or burst frequency; the other is the spike frequency, corresponding to the frequency with which the fast sodium spikes repeat inside the burst. These two frequencies were measured in model neurons using a method conceptually similar to autocorrelation (see Appendix 1 for implementation details). An auto-distance value \(A(\tau) = D_W(r(t), r(t+\tau))\) was calculated over \(0 \leq \tau < 5\); \(A(\tau)\) was lowest when the waveforms overlapped best. \(A(\tau)\) was mean filtered and one or two minima were detected, the larger corresponding to burst frequency, the smaller to spike frequency. In non-spiking neurons, only one frequency was detected.

Distance metric calculations

Various distance metrics were constructed to compare the output of two models with different parameter value vectors \(p_1\) and \(p_2\). The waveform distance, \(D_W(p_1, p_2)\) was calculated as usual, i.e., the area between the response waveforms:

\[
D_W (r_1, r_2) = \frac{1}{d} \left( \int_0^d |r_1(t) - r_2(t)|^p \right)^{1/p}
\]

where \(r_1\) and \(r_2\) are the response waveforms of the models generated by \(p_1\) and \(p_2\), respectively, \(d\) is the total duration, and \(p = 1\). Our rhythmic model neurons produced many burst cycles in the long duration (60 s or 174 s) used here. When used to measure the distance between periodic waveforms with many cycles, the \(D_W\) metric becomes highly sensitive to frequency differences (Figure 2), and relatively insensitive to features such as spike timing or number or waveform shape. Thus \(D_W\) functioned primarily as a slow-wave frequency difference detector in this work.
Waveform distance \( D_w \) between two 60 s sine waves, \( f_0 = 1 \text{ Hz} \), and \( f_1 \) ranging from 0.9 Hz to 1.1 Hz. As frequency diverges, \( D_w \) rapidly increases, with a characteristic ripple pattern.

**Figure 2.** Sensitivity of \( D_w \) distance metric to frequency differences with long samples.

\( D_w \) satisfies the four criteria for a metric space:

1. \( D_w(\mathbf{r}_1, \mathbf{r}_2) \geq 0 \) (non-negativity)
2. \( D_w(\mathbf{r}_1, \mathbf{r}_2) = 0 \) if and only if \( \mathbf{r}_1 = \mathbf{r}_2 \) (identity)
3. \( D_w(\mathbf{r}_1, \mathbf{r}_2) = D_w(\mathbf{r}_2, \mathbf{r}_1) \) (symmetry)
4. \( D_w(\mathbf{r}_1, \mathbf{r}_3) \leq D_w(\mathbf{r}_1, \mathbf{r}_2) + D_w(\mathbf{r}_2, \mathbf{r}_3) \) (triangle inequality)

Satisfaction of the first three criteria follows directly from the definition of waveform distance, and waveform distance has been shown to satisfy the triangle inequality (Rynne BP and Youngson MA, 2000).

The phase plane distance, \( D_p(\mathbf{p}_1, \mathbf{p}_2) \), was calculated by resampling the two response waveforms at 1 ms intervals and binning the voltage and \( dV/dt \) values into 256 x 256 density matrices, scaled to cover the entire \( V \) and \( dV/dt \) range of both response waveforms. The absolute difference in count between every corresponding set of entries among the two matrices was
summed, with a sum of 0 indicative of complete overlap (LeMasson G and Maex R, 2001). The $D_p$ metric primarily identified waveforms with similar slow wave shape, and was insensitive to phase differences and considerably less sensitive to frequency differences than waveform distance. This metric fails the identity criterion and thus does not satisfy the requirements for a metric space.

**Parameter ranges and scaling factors**

The parameter space of the Prinz mathematical model is infinite in extent. In practice, however, $\bar{g}$ (maximal conductance) values below 0 or above certain amounts are physiologically nonsensical. Thus, the various $\bar{g}$ parameters were bounded between 0 and an upper bound appropriate to the conductance, within an order of magnitude greater than values previously reported for pyloric neurons. Based upon initial explorations of parameter space, these upper bound values of some conductance parameters were adjusted by multiplying or dividing by 2 until the parameter space was adequately sampled.

The Prinz model is more sensitive to changes in some parameters than others. For example, in current clamp, very small changes to $\bar{g}_H$ elicit large changes in model output, whereas large changes to $\bar{g}_{kad}$ are typically required to elicit similarly large output changes. To allow the flood-fill algorithm to explore parameter space with fixed step sizes despite the model’s different sensitivity to different parameters, scaling factors were calculated (*Figure 3*). A random point $p$ in parameter space was chosen, and a 60 s waveform response was generated for that set of parameters using the phase-locking current pulse perturbation. Then, for each of the eight $\bar{g}$ parameters, small regular steps were taken, increasing or decreasing the value of that parameter away from the original point. After each step $\Delta p$, a waveform response was generated for the new parameter point $p + \Delta p$, and the steps were continued until $D_w(p, p + \Delta p)$ exceeded a threshold ($\varepsilon = 5$). A new random point $p$ in parameter space was chosen, and the process repeated for a
total of 1000 points. For each parameter, the mean of all 2,000 distances (1,000 made with increasing steps, 1000 with decreasing steps) was calculated; this was then used as an approximate scaling factor. These scaling factors were adjusted as needed during the flood fill operation if it appeared that a parameter dimension was under- or over-sampled. These scaling factors and upper and lower bounds are reported in Table 3.

*Figure 3. Scaling factor calculation*

Initial point \( p \) in parameter space (filled circles) was chosen, and response was generated for \( M(p) \). Parameters were changed (\( \Delta p \)) along basis vectors (dotted line arrows) until waveform distance \( D_w(M(p), M(p + \Delta p)) \) exceeded \( \varepsilon = 5 \) (black boundary shapes). Mean (\( \mu \)) of \( \Delta p \) for each parameter was used as the scaling factor.

*High-performance computing*

Where possible, algorithms were parallelized and tasks distributed on an in-house high-performance computing cluster of 8 hosts running Fedora Linux, 8 cores per host, 2 threads per
core (Advanced Clustering Technologies, Inc). OpenMPI was used as the communications layer, with additional custom infrastructure software written in C and Perl.

Table 3
Scaling Factors and Bounds for Prinz Model Parameters

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<th>Current</th>
<th>Leak</th>
<th>CaT</th>
<th>CaS</th>
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</table>

Parameter-space raster scans

The rasterscan program was written and used for the coarse grid searches (raster scans) of parameter space slices around a reference point \( p_{\text{ref}} \). Two-dimensional scans were performed by iterating the values of two out of the eight parameters across a plane at 256 regular intervals, for a total of \( 256^2 = 65,536 \) total points. All other parameter values were held constant and equal to the corresponding values in \( p_{\text{ref}} \). At each raster-scanned point \( p \), one or more distance metric values \( D_X(p, p_{\text{ref}}) \) were calculated and recorded. Spike and burst frequencies (Materials and Methods - Frequency detection) were also detected and recorded for the model characterized by \( p \). The resulting data were saved to files. The raster scan was then repeated for all other combinations of two out of the eight parameters. Three-dimensional volume explorations were conducted similarly, by iterating three of the eight parameters of \( p \) at 64 regular intervals each \( (64^3 = 262,144 \) total points), recording distance metric values and frequencies, repeating for all possible combinations of parameters.
The resulting data files were typically rendered as surfaces (two-dimensional scans) or volumes (three-dimensional scans). For surfaces, the $x$ and $y$ coordinates corresponded to the two parameters being scanned, and a distance metric value or frequency was selected for the $z$ coordinate. In some plots, a second dependent variable was used to select a color for the point. For three-dimensional volume scans, the $x$, $y$, and $z$ coordinates corresponded to the parameters being raster scanned, and the distance metric or frequency was rendered as the color for that point. In most three-dimensional visualizations, only those points whose dependent variable (and thus color) was within a desired range were rendered; with others omitted for visual clarity.

*Vector / flood-fill basin explorations*

Though useful for visualizations, the raster scan process is limited in three important ways. First, it cannot explore outside of the arbitrary bounds specified for the scan. Second, it will not find details which are finer than the resolution of the grid specified. Third, it explores the entire range specified, which is computationally wasteful if the goal is to investigate only those points within the basin of equivalence. To address these limitations, a second process was used to explore the basins of equivalence in high detail, and potentially find areas which could not be reached by the raster scan process. This was done first by exploring random vectors starting from $p_{\text{ref}}$ to produce seed data, and then stepping outward from this seed data at regular intervals until the boundary of the basin of equivalence was reached.

To produce the seed data, the *vballoon* (vector balloon) was written. This program built and maintained a set of line segments in parameter space which were within the basin of equivalence. Initially, this set contained only the zero-length line segment $(p_{\text{ref}}, p_{\text{ref}})$. Repeatedly, the program picked a line segment at random from the set, then chose a random point $p$ along it. Then, it started moving along a random vector $A_p$ from that point, stopping once the boundary of the basin of equivalence was reached, i.e., $D_W(p + A_p, p_{\text{ref}}) \geq \varepsilon$. The steps taken were initially
small, and increased in size until the boundary was reached; at that point, step size was decreased, until a point was found within the basin of equivalence, or the original step size was reached. This varying step size allowed the program to rapidly find the boundary with high accuracy. The new line segment \((p, p + \Delta p)\) was then added to the set of line segments, and the process was repeated (Figure 4). Several optimizations were implemented to improve performance by biasing the random selections; for details see Appendix 1.

![Figure 4. Operation of vballoon program](image)

Boundary of basin of equivalence (blue) was found by random vector explorations (black arrows) from areas previously found within the basin, starting with the reference point \(p_{\text{ref}}\) (blue dot). A point along a random vector was chosen, and increasing size steps taken (green dots), until the boundary was exceeded (red dot). The step size was then decreased (red stars) until the point was within the boundary (green star).
The seed points (starting and ending points of all line segments) were then passed to the cloud_mesh program. This program started by aligning each seed point on a regular, high-resolution grid. As it was possible that this would move a point originally within the basin of equivalence to a location outside it, the waveform distance was recalculated for each newly “grid-snapped” point. Those which were outside the basin after grid alignment were rejected and analyzed separately (see below). The grid-aligned points were then used as the starting points for a flood-fill process, repeatedly testing all unexplored adjacent neighbors (e.g., 6 total in 3-space slices) to known points within the basin until the region was filled (Figure 5, 6).

The seed points which were not within the basin of equivalence after the grid alignment were tried again on a grid of twice the resolution. This was repeated at 4x, 8x, and 16x resolution, until the point was either within the basin, or all resolutions had been tried, at which time the point was discarded. The flood fill process was then repeated on these finer resolution grid-aligned seed points, until the edges of the coarser grid were reached. In this way, high resolution data were obtained for regions along the boundary of the basin of equivalence.
Figure 5. Operation of cloud\_mesh program

Seed points (black dots) from the vballoon exploration vectors (black arrows) were snapped to a grid. Those which remained within the boundary of the basin of equivalence (green dots) were kept; those outside (red dots) were analyzed separately. The adjacent points were then tested; those within the basin (green circles) were kept for the next iteration of the flood fill, and those outside (red circles) rejected. The flood fill continued outward until all points within the basis were tested.
Figure 6. Flood-fill exploration of basin of equivalence in a slice of parameter space.

In all panels, the X, Y, and Z axes correspond to \( \overline{g_{CaS}} \), \( \overline{g_{CaS}} \), and \( \overline{g_{KCa}} \), respectively. A-C: vector explorations of the slice of parameter space after 100 (A), 1000 (B), and 10000 (C) program iterations. By chance the initial vectors mostly explored a planar region; later vectors reached the curved section extending into increasing \( \overline{g_{KCa}} \). D: flood fill results utilizing the seed points obtained from vector explorations.
Minkowski-Bouligand dimension calculations

The Minkowski-Bouligand (box-counting) dimension, $\text{Dim}_{\text{box}}$, of a basin of equivalence in an $n$-dimensional parameter space was calculated to assess the morphology of the basin, both in three-dimensional slices and in the complete set (Falconer, 2003). Initially, one $n$-dimensional box was constructed of size required to cover the entire set of points in the basin of equivalence. This box was then divided in half along each axis for a total of $2^n$ boxes; the total number of those boxes required to cover the set, $N(2)$, was recorded. This was repeated for divisions into thirds for $3^n$ boxes and the total, $N(3)$, required to cover the set was recorded. This process was continued, recording $N(k)$ for $k$-fold divisions of the original box into $k^n$ boxes, until the divisions were smaller than the original grid size used for exploring the basins of equivalence. When plotted in log scale, as $\log(N(k))$ vs $\log(k)$, the curve converges to a line whose slope is the Minkowski-Bouligand dimension, before diverging due to undersampling (i.e., where the Minkowski-Bouligand box size approached the original flood-fill grid size).

To properly select the region of the curve for measuring dimension, and avoid measuring dimension where undersampling was a concern, the box counts were calculated twice. The first counts were performed on only the flood fill points which aligned to the grid. The second count was performed with the additional points which required a higher resolution grid (Vector/flood-fill basin explorations). Regions for fitting were selected visually, excluding the areas where these two curves diverged (Figure 7).
Figure 7. Minkowski-Bouligand dimension estimation.

Number of boxes of $k$-fold divisions per axis, $N(k)$, required to cover a basin of equivalence was counted. Counts were made without (red) and with (green) the additional points which the flood-fill algorithm aligned to a finer grid. Linear fits were made to the first two counts over $3 \leq \log(k) \leq 4$ to obtain a dimension estimate.

Visualization

Surface and volume data were rendered using the gnuplot graphing program. Where the number of data points in a set to be rendered was greater than 400,000, the set was downsampled by first eliminating all interior points and then randomly sampling, without replacement, at most 400,000 points to display. When surface grids containing frequency information were visualized,
the frequency values were smoothed by mean filtering with three passes, to reduce the appearance of noise introduced by the frequency detection algorithm.

Statistical analyses

Most statistical analyses were performed using the public domain statistics language R and its packages (r-project.org). Correlation tests were performed using the `rcorr` function in R to calculate Pearson's correlation coefficients, with significance. The `cordif.dep` function of the `multilevel` package in R was used to assess whether two correlation coefficients differed significantly (Cohen J and Cohen P, 1983). The Holm-Bonferroni method (Aickin and Gensler, 1996) was used to adjust $p$-values when multiple hypothesis tests were conducted on the same data. Linear regression models were performed using the `lm` function in R.
Results

Raster scan surfaces and volumes

All two-dimensional slices of the Prinz model parameter space were raster scanned (Materials and Methods - Parameter-space raster scans) in the region around the \( p_{\text{ref}} \) reference model, with a 174 s simulation time. At each point, the model’s burst and spike frequencies were calculated, and \( D_w \) (waveform distance) and \( D_p \) (phase plane distance) versus the \( p_{\text{ref}} \) model were calculated. The data were then visualized as difference landscape surfaces. Except for those slices in which \( g_{\text{leak}} \) was varied, in which small changes in leak current rapidly terminated rhythmic behavior, the \( D_w \) surfaces all contained pronounced low-\( D_w \) regions extending throughout the scanned region of the slice (ridges and peaks in Figure 8; note reversed z axis). Within these regions, the corresponding model neurons’ output waveforms were similar in frequency, phase, and overall shape. The ridges usually appeared continuous. Occasionally, multiple apparently disjoint ridges were observed, possibly connected in an unvisualized dimension (Figure 8C); model neurons from disjoint ridges did not obviously differ in activity. Ridges were occasionally flanked by the ripples characteristic of the \( D_w \) metric’s response to frequency differences (Figure 8 B,D; compare Figure 2). When surfaces were visualized with height corresponding to burst frequency, low \( D_w \) regions often had similar frequency (Figure 9 A,B).

The \( D_p \) difference landscapes had a similar appearance to the \( D_w \) landscapes, i.e., pronounced ridges which extended throughout the parameter space (Figure 10). However, the regions whose models were similar to the reference by the \( D_p \) metric were not the same as those that were similar by the \( D_w \) metric, and in some cases they appeared nearly orthogonal (Figure 10 A,D). The phase plane distance metric was clearly sensitive to different features of the waveform.
compared to the waveform distance metric, though, in both cases, only a narrow band of models were scored as being similar to the reference model.

To further determine the extent of the basins of equivalence, the rasterscan program was run for three-dimensional slices of parameter space using the same reference model, and the resulting points for which $D_w < \varepsilon$ were selected. A threshold of $\varepsilon = 11$ was chosen to correspond with the observed $D_w$ values within the ridges on the two-dimensional difference landscapes. Visualization of these 3-space regions revealed that the $D_w$ basins of equivalence were not simply two-dimensional structures, but were extensive in at least three dimensions, appearing as a curved sheet (Figure 11). Thus, at least three parameters could covary without appreciably changing model output. The basins of equivalence for the phase plane histogram distance metric, $D_p$, were similarly visualized in three dimensions, with a threshold $\varepsilon = 0.75$ which corresponded with the ridges in the two-dimensional surfaces. Again, the basins of equivalence appeared in three-dimensional slices as curved regions (Figure 12).

Similar examinations were made using other reference model neurons, in both two and three-dimensional slices of parameter space. The morphology and extent of the basins of equivalence were similar to those observed with the previous reference model, and no additional insights were obtained.
Figure 8. Raster scanned $D_w$ difference landscapes in the Prinz model

Four of the 28 total parameter space slice landscapes are shown. In each, two maximal conductance parameters were varied, while all others were held identical to the reference model’s parameters. Height and color both correspond to $D_w$. To improve visibility, $z$ axis is reversed; dark peaks and ridges are regions whose models produce similar output to the reference (low $D_w$). Due to the sensitivity of $D_w$ to frequency differences with long samples (174 s), these models are essentially identical in frequency. Models outside the peaks differ from the reference in frequency, amplitude, or other features. Ripples (lower right edge of B and D) appear due to the characteristic response of $D_w$ to frequency changes with long samples (Figure 2).
Figure 9. Raster scanned slow-wave frequency landscapes

A-D: Parameter slices corresponding to those in Figure 8. Height corresponds to slow wave frequency, with $z$ axis reversed; non-oscillating models were assigned a height of 0. Color corresponds to $D_w$. The dark purple ribbons, where $D_w$ is low, generally have similar height, and thus frequency. Not all models which are isochronous with the reference will necessarily have low $D_w$, and thus while $D_w$ is most sensitive to slow wave frequency, other features of model activity also affect it.
Figure 10. Raster scanned $D_p$ difference landscapes

A-D: Parameter slices corresponding to those in Figure 8. Height corresponds to phase plane distance ($D_p$), with $z$ axis reversed and in log scale for improved visibility. Color corresponds to $D_w$ (height and color in Figure 8). The $D_p$ basins of equivalence (peaks and ridges) do not correspond to the $D_w$ basins of equivalence (dark blue ribbons), at times being nearly orthogonal (A,D); using both metrics in parameter fitting could thus be advantageous. Nonetheless, the regions of low $D_p$ have a similar appearance, being relatively thin and extending throughout the parameter space slice.
Figure 11. Three-dimensional raster scans using $D_W$ metric.

Four of the 56 total parameter space 3-slices landscapes are shown. Only those points for which $D_W < 11$ (for simulation time of 174 s) are shown. Points are colored by $D_W$ value. As with the two-dimensional slices, the basins of equivalence are relatively thin regions which curve and extend throughout the parameter space.
Figure 12. Three-dimensional raster scans using $D_P$ metric.

A-D: Three-dimensional parameter space slices as in Figure 11. Only those points for which $D_P < 0.75$ (with a simulation time of 174 s) are shown. Points are colored by $D_W$ value; note that not every point with a low $D_P$ also has a low $D_W$ value (dark points), thus, the low-$D_P$ regions do not correspond to the low-$D_W$ regions. As with the $D_W$ basins of equivalence, the $D_P$ basins appear to be sheet-like.
Sodium-blocked surface and volume raster scans

The spontaneous activity of the pacemaker reference neuron exhibited both slow-wave and spiking activity. Having ascertained the existence of sheet-like basins of equivalence in the full parameter space, we wanted to see whether they would remain with the sodium conductance removed. This would give results applicable to ongoing in vivo pyloric neuron research in our lab, in which the fast sodium conductance is blocked with TTX. Furthermore, it could simplify future stages of research by reducing the computational demands needed to investigate the shape of the basins of equivalence. We therefore repeated the raster scans in a reduced 7-dimensional parameter space with sodium removed, using as a reference point the original \( p_{\text{ref}} \) without sodium. The removal of the fast sodium conductance eliminated nearly all of spikes from the reference model, leaving behind only a calcium spike. With the spike trains gone, the duration of both the depolarized plateau and the hyperpolarized phase decreased (Figure 13). Surfaces were rendered as \( D_W \) difference landscapes (Figure 14), \( D_P \) difference landscapes (Figure 15), and frequency landscapes (Figure 16). Three-dimensional scans were also conducted (Figure 17).

Figure 13. Reference model neuron with and without fast sodium conductance.

Again, extensive basins of equivalence were observed in slices of parameter space as ridges ranging throughout the scanned region. Without the fast sodium spike trains, the ridges appeared more continuous and better defined. The $D_w$ and $D_P$ basins were again generally independent and often intersected only in the region of the reference model.

Figure 14. Raster scanned $D_w$ difference landscapes, without sodium conductance. Four parameter space slices are shown, as in Figure 8; note that parameters in panel B differ from those in Figure 8. Height and color both correspond to $D_w$, with $z$ axis reversed. Basins of equivalence were clearly visible as ridges, with the characteristic ripples due to the response of $D_w$ to frequency shifts (Figure 2) sometimes appearing (C, D).
Figure 15. Raster scanned $D_F$ difference landscapes, without sodium conductance

Four parameter space slices are shown as in Figure 10, but without the sodium conductance; again, parameters in panel B differ from Figure 10. Surfaces are colored by $D_W$. As is the case with the sodium conductance present, the $D_F$ basins of equivalence are independent of, and sometimes (A, D) orthogonal to, the $D_W$ basins of equivalence. The $D_F$ landscapes appear very similar to those in which sodium is present (compare Figure 10 A, C, D), suggesting that $D_F$ is most sensitive to slow wave shape.
Figure 16. Raster scanned frequency landscapes, without sodium conductance

Four parameter space slices are shown as in Figure 9, but without the sodium conductance; again, parameters in panel B differ from Figure 9. Height corresponds to frequency with z axis reversed, color to D_w; again the low-D_w regions (dark blue) exist in a narrow frequency band, appearing at a similar height in the figures.
Figure 17. Three-dimensional raster scans using $D_w$ metric, without sodium conductance

Four of the 56 total parameter space 3-slices landscapes are shown. Only those points for which $D_w < 11$ (simulation time 174 s) are shown. Points are colored by $D_p$ value. Note that, within the low $D_w$ region shown, typically only a small area (dark blue) also has low $D_p$ value.
High-resolution flood fill explorations

The previously conducted raster scans of slices of parameter space, though relatively coarse, revealed that basins of equivalence were present with complex morphology. Furthermore, such regions were present even when the sodium conductance was removed from the model. To further characterize the nature of these regions, we utilized the vballoon and cloud_mesh programs (Materials and Methods - Vector / flood-fill basin explorations) to explore the $D_w$ basins of equivalence in high resolution in the sodium-blocked model, without leak present. This was first done in all three-dimensional slices of the reduced 6-dimensional parameter space. Then, a final exploration was made of the 6-dimensional parameter space. Repeated vector explorations were conducted with the vballoon program to obtain sufficient seed data. To facilitate finding regions of parameter space which could conceivably be connected through physiologically nonsensical conductance values in the dimensions being explored, parameter values were not constrained during the generation of this seed data, and parameter values with negative maximal conductances were in fact identified within the basin of equivalence (note, however, that regions within the basin of equivalence which were not connected in the dimensions being explored could not be reached). These data were then provided to the cloud_mesh program which aligned them to a high-resolution grid and performed a flood-fill algorithm to completely explore the region bounded by $D_w<\varepsilon$. To improve algorithm performance, simulations were run for 60 s instead of 174 s, with $\varepsilon$ adjusted accordingly, again discarding the first 5 s including the response to the phase-locking pulse. Example 3-space slice basins of equivalence are shown in Figure 18.
Figure 18. High resolution three-dimensional $D_W$ basins of equivalence

Four 3-slice basins of equivalence are shown. Basins of equivalence appeared as thin sheets, often highly curved. Gaps were observed within some sheets; these may occur due to the sheet curving into an unvisualized dimension. Edges may represent regions where the sheet curves into an unvisualized dimension, where the waveform distance increases past the threshold, or where the exploration bounds are reached. Points are colored by $D_W$ value. The fringe-like appearance of some edges (D) and the moiré pattern appearance (A) are artifacts of the flood-fill grid.
Visualization of the slices again revealed curved regions of space within which $D_w$ remained low. The basins of equivalence were typically curved, often highly so. There were visible voids (not shown), consistent with the sheet curving into a higher (unvisualized) dimension. Edges to the sheet-like regions occurred where the flood-fill was bounded by the exploration limits (Table 3), where the models’ outputs varied too greatly from the reference, or where the sheet curved into higher dimensions. The latter condition sometimes produced a fringe-like appearance where the portion of the sheet remaining in the 3-space slice became thin, revealing the underlying grid resolution.

*Extents and fractal dimensions*

The maximum and minimum values of each parameter were measured throughout all explored regions, both the 3-dimensional slices and the full 6-dimensional basin. These are reported in Table 4. In many cases the predefined exploration limits were reached, particularly at the lower bound.

Table 4

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CaT</th>
<th>CaS</th>
<th>A</th>
<th>KCa</th>
<th>Kd</th>
<th>H</th>
</tr>
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<tbody>
<tr>
<td><strong>Min</strong></td>
<td>0 *</td>
<td>0 *</td>
<td>0 *</td>
<td>2.16</td>
<td>0 *</td>
<td>0.0056</td>
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<tr>
<td><strong>Max</strong></td>
<td>41.28</td>
<td>20.4</td>
<td>100 *</td>
<td>50 *</td>
<td>500 *</td>
<td>0.02 *</td>
</tr>
</tbody>
</table>

Minima and maxima are for all explored slices of parameter space together. All $\bar{g}$ values in mS/cm$^2$. (*) indicates the exploration limits were reached.
To estimate the morphological nature and complexity of the reduced-dimension sets and of the full 6-dimensional basin of equivalence, the fractal dimension $\text{dim}_{\text{box}}$ of each set was calculated utilizing the box-counting method (Materials and Methods - Minkowski-Bouligand dimension calculations). The fractal dimensions of the basins of equivalence in the 3-dimensional slices, and in the full 6-dimensional exploration, ranged between 1.8 to 2.1, typically close to 2.0 (Table 5).

Table 5

Minkowski-Bouligand Dimension Estimates of Basins of Equivalence

<table>
<thead>
<tr>
<th>Slice</th>
<th>$\text{Dim}_{\text{box}}$</th>
<th>SE</th>
<th>Slice</th>
<th>$\text{Dim}_{\text{box}}$</th>
<th>SE</th>
</tr>
</thead>
<tbody>
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<td>$\text{CaT, CaS, A}$</td>
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<td>0.0040</td>
<td>$\text{CaT, CaS, KCa}$</td>
<td>1.8</td>
<td>0.0032</td>
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<tr>
<td>$\text{CaT, CaS, Kd}$</td>
<td>2.1</td>
<td>0.0055</td>
<td>$\text{CaT, CaS, H}$</td>
<td>1.9</td>
<td>0.0043</td>
</tr>
<tr>
<td>$\text{CaT, A, KCa}$</td>
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<td>0.0050</td>
<td>$\text{CaT, A, Kd}$</td>
<td>2.1</td>
<td>0.0042</td>
</tr>
<tr>
<td>$\text{CaT, A, H}$</td>
<td>2.0</td>
<td>0.0042</td>
<td>$\text{CaT, KCa, Kd}$</td>
<td>2.0</td>
<td>0.0028</td>
</tr>
<tr>
<td>$\text{CaT, KCa, H}$</td>
<td>1.9</td>
<td>0.0039</td>
<td>$\text{CaT, Kd, H}$</td>
<td>2.0</td>
<td>0.0033</td>
</tr>
<tr>
<td>$\text{CaS, A, KCa}$</td>
<td>1.9</td>
<td>0.0054</td>
<td>$\text{CaS, A, Kd}$</td>
<td>2.1</td>
<td>0.0026</td>
</tr>
<tr>
<td>$\text{CaS, A, H}$</td>
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<td>0.0029</td>
<td>$\text{CaS, KCa, Kd}$</td>
<td>1.9</td>
<td>0.0034</td>
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<td>$\text{CaS, KCa, H}$</td>
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<td>0.0056</td>
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<td>0.0025</td>
<td>$\text{A, KCa, H}$</td>
<td>2.0</td>
<td>0.0029</td>
</tr>
<tr>
<td>$\text{A, Kd, H}$</td>
<td>2.0</td>
<td>0.0023</td>
<td>$\text{KCa, Kd, H}$</td>
<td>2.0</td>
<td>0.0023</td>
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<tr>
<td>$\text{CaT, CaS, A, KCa, Kd, H}$</td>
<td>2.1</td>
<td>0.0751</td>
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</table>

SE = Standard Error.
To confirm that the $D_W$ distance metric was sensitive to slow-wave frequency, a random sample of 1,000 entries was taken without replacement from among the raster scanned parameter space data. Each entry consisted of the parameter space point as well as the calculated $D_W$, $D_P$, slow-wave frequency, and spike frequency. Frequency differences versus the reference model were calculated for each entry to produce a slow-wave frequency difference and a spike frequency difference. A correlation test was then performed for $D_W$ against both slow-wave and spike frequency differences. $D_W$ correlated significantly with slow-wave frequency difference ($R=0.54$, $p<0.05$), but did not correlate significantly with spike frequency difference ($R=0.05$).
Discussion

Previous research has established that multiple combinations of $\bar{g}$ parameter values can give rise to pyloric model neurons which, under free-running conditions, behave similarly (Achard and De Schutter E., 2006; Prinz et al., 2004). Qualitatively similar behavior has been demonstrated across a wide range of parameter space, with different parameter values yielding neurons which behaved similarly, i.e., bursting, spiking, or quiescent. Quantitatively similar behavior has also been demonstrated across different regions of parameter space, with model neurons with different parameter values producing similar output as measured by $D_P$ (Achard and De Schutter E., 2006). The goal of this research was to characterize the morphology and extent of these regions, i.e., the basins of equivalence, in the Prinz model. If these regions are extensive throughout parameter space, then activity cannot be used to determine parameter values. If the basins are continuous, then for any two neurons with similar activity, neuronal parameter values of one neuron can covary to become the other without changing activity. We chose to assess similarity in the Prinz model against a reference model neuron from previously published work, because this neuron produced rhythmic output which was typical of real pyloric neurons, and because repeating these assessments with other reference model neurons did not produce additional insights.

Central to characterizing the basins of equivalence is the choice of how, and against what, to measure similarity. We chose to assess similarity using two distance metrics, waveform distance ($D_W$) and phase plane distance ($D_P$). Both are commonly used for this purpose. Waveform distance is particularly easily understood, and has the required characteristics to define a metric space, which is advantageous for analytical purposes. With long duration samples, it is especially sensitive to slow-wave frequency difference, while being relatively insensitive to spike frequency. Phase plane distance is especially sensitive to slow-wave shape, is less sensitive to
frequency, and produces difference landscapes which are similar in the presence or absence of the fast sodium conductance (compare Figure 12 and Figure 15).

We then explored broad regions of parameter space in two- and three-dimensional slices. Extensive basins of equivalence were observed regardless of which distance metric was used, though they differed in location and shape. Such regions were observed despite changing the reference model neuron, and whether the fast sodium current was present or absent. For the $D_W$ metric, they contained model neurons producing output which was largely isochronous to the reference model. However, the model neurons in the $D_W$ basins also had outputs of similar amplitude, phase, shape, and other characteristics that appeared visibly similar (Figure 19). Model neurons in the $D_P$ basins did not necessarily have the same frequency, but did have similar slow wave activity (Figure 20).

Because basins of equivalence were observed regardless of the metric used or whether the fast sodium conductance was present, we chose to perform high-resolution flood fill explorations in a reduced parameter space excluding the leak and fast sodium conductances, using the $D_W$ metric. The former was not present in our reference model neuron, and its addition rapidly resulted in increased distance measures by all metrics. The latter was not necessary for extensive basins of equivalence to be revealed, and removing it made the results more applicable to ongoing in vivo experiments in our lab.

When visualized in high resolution in reduced parameter slices, the $D_W$ basins of equivalence appeared as curved sheets (Figure 18). Their Minkowski-Bouligand dimensions were always close to 2.0 (Table 5), which is consistent with a sheet-like object. The full six-dimensional basin of equivalence had a similar Minkowski-Bouligand dimension of 2.1, suggesting that the basin is a curved hypersheet. Further supporting this conclusion is that the edges of the sheet apparently vanished in a way consistent with curvature into higher dimensions,
thinning out and appearing fringe-like or showing voids or gaps where bulges might occur. On this basis, we conclude that the basin of equivalence is likely a highly curved hypersheet which extends throughout parameter space.

Although visualization of a full six dimensions is difficult or impossible, it is possible to gain some visual insights from the 6-dimensional basin of equivalence set. By mapping three of the parameters to \((x,y,z)\) coordinates and a fourth to color, it is possible to “project” the full set into three dimensions in a way that suggests curvature in an additional dimension (Figure 21). Within the curved hypersheet, the model neurons’ behavior remains similar, most notably in (but not limited to) slow wave frequency. As parameters covary along the local curvature of the sheet, the model’s output will not change appreciably. As they vary orthogonally to the sheet’s local curvature, however, the model output will rapidly change in frequency or another characteristic. This has several important implications. First, because the sheet is highly curved, occasionally doubling back on itself, regulatory feedback mechanisms which are based upon simply increasing or decreasing particular maximal conductance parameters in response to output may not be feasible. For example, in the basin of equivalence in Figure 18C, when \(\bar{g}_{Ca}\) is high (near 12 mS/cm²), constant activity is maintained by increasing \(\bar{g}_A\) when \(\bar{g}_{Ca}\) is below 8 mS/cm², but when \(\bar{g}_{Ca}\) is above 8 mS/cm², activity is maintained by decreasing \(\bar{g}_A\). Thus, the neuron could not maintain its activity by simply increasing \(\bar{g}_A\) when slow-wave frequency is too low.

Second, the relative thinness of the sheet suggests that, when attempting to combine parameters measured from multiple experiments, it should be expected that the composite model’s output will be different the original neurons. It is highly unlikely that one could sample different parameters from various locations of the sheet, combine those parameters together, and still wind up within the sheet. Were the basin of equivalence more blob-like, this might be more feasible.
Figure 19. Five model neurons from the DW basin of equivalence

Five points were randomly selected from the 6-dimensional parameter space flood fill exploration performed of the basin of equivalence. Despite being located in different regions of parameter space, the model outputs have nearly identical frequency. Slow wave shape is less conserved (compare D and E).
Figure 20. Five model neurons from the \( D_p \) basin of equivalence

Five points of low \( D_p \) value were randomly selected from the collection of 3-dimensional parameter space slice raster scans performed of the \( D_p \) basin of equivalence, without the fast sodium conductance. The slow wave shape is similar among these neurons despite differing frequency.
A final concern applies to methods which attempt to characterize the parameters of a neuron (model or not) based upon its output. One method for characterizing multiple conductance parameters is mathematical optimization, adjusting parameters of a model neuron until the output of the model matches the reference waveform, typically using appropriate heuristic methods such as simulated annealing or an evolution strategy (Hobbs and Hooper, 2008). The existence and extent of the basins of equivalence, and the relatively pathological curvature of the parameter surfaces, are a challenge to fitting models to the output of free-running live or model neurons. In particular, $D_w$ when used with long sample times produces regions of low fitness adjacent to the basins of equivalence, and these may impair convergence of such heuristics. Additionally, the adjacent ripples may also produce local minima in which the algorithm could become trapped. Even when the heuristic does find its way into the basins of equivalence, their extent throughout parameter space presents a difficulty to finding a unique

Figure 21. Projection of 6-dimensional $D_w$ basin of equivalence into four dimensions. Points from the 6-dimensional basin of equivalence were rendered by converting four of the parameters (maximal conductance values for $C_{aT}$, $C_{aS}$, $K_{Ca}$, and $H$) to $x$, $y$, $z$, and color, respectively; $A$ and $K_d$ maximal conductance values varied, but were not plotted.
solution. The use of multiple distance metrics is a potential means to address some of these concerns, as the $D_p$ basins do not generally coincide with the $D_w$ metric basins. A fitness function which utilizes both metrics could thus offer better optimization performance than one which relies upon waveform distance alone.

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CHAPTER 3: EMPIRICALLY OPTIMIZED PERTURBATIONS CHARACTERIZE AND SEPARATE NEURON MODELS

Author Contributions

The research in this chapter was initiated in an attempt to understand and resolve difficulties with ongoing research in the S. L. H. lab attempting to fit a neuron model to the perturbed activity of pyloric neurons. W. E. W. analyzed the properties of the white noise perturbation in the model, and designed and carried out *in silico* experiments to identify more appropriate perturbations. During this research, W. E. W. developed a procedure to empirically design a perturbation using a genetic algorithm, and included this perturbation in the experiments. W. E. W. wrote all software and performed all experiments. S. L. H contributed to research design and interpretation of results. S. L. H. and all dissertation committee members assisted in editing the manuscript and figures, with W. E. W. making revisions according to their feedback.

Abstract

Prior work has established that conductance based neuron models can produce similar output with widely different parameter values. When appropriately perturbed, the responses of these neurons can however separate, and may provide enough information in their responses to allow fitting of parameters using heuristic methods. An evolution strategy is used to design a voltage clamp perturbation for a pyloric neuron model which activates each voltage-dependent current against the background of other currents. This perturbation exceeds the performance of traditional voltage clamp perturbations in causing the responses of model neurons to vary with changes to any parameter value, and facilitates the use of heuristic methods for parameter fitting. Similar tests conducted in current clamp show that noise-based perturbations with time-varying bandwidth offer superior performance at these tasks.
Introduction

Previously, it was shown that many different combinations of parameter values in the Prinz model can generate model neurons which produce nearly identical output waveforms in free-running conditions. The parameter space of this model – the set of all possible combinations of parameter values – contains thin but extensive and highly curved sheet-like regions, the basins of equivalence, in which the corresponding models behave similarly to an arbitrary reference model. Such widespread basins exist whether similarity is assessed using waveform distance ($D_w$) or phase plane distance ($D_p$), whether the model includes the fast sodium conductance or not, and regardless of the choice of reference model. An equivalent phenomenon has been noted in real neurons, in which similar activity can be occur with different parameters (Desai et al., 1999; Golowasch et al., 1999; Haedo and Golowasch, 2006; LeMasson et al., 1993; Li et al., 1996; Liu et al., 1998; MacLean et al., 2003; Marder and Prinz, 2002; Marder and Goaillard, 2006; Mizrahi et al., 2001; Turrigiano et al., 1995).

A common goal in neuroscience research is constructing a computational model of a live neuron which reproduces its activity. The traditional method for doing so involves applying chemical blockers to isolate a specific conductance, using voltage clamp step protocols to test the voltage-dependence of the conductance and its dynamics, and then fitting the neuron’s responses to suitable mathematical models to recover its conductance parameters. As these chemical blockers are often irreversible, measurements must be taken across several neurons before a model can be constructed. Even when these neurons have identical electrical activity, however, it is possible that they may have different conductance parameters. Accordingly, when such models are actually constructed from measurements made in several experiments, they often produce incorrect output, and their conductance parameters must be manually adjusted. This is unsurprising if the sheet-like topology of the basins of equivalence are representative of the
dynamics of real neurons; even a slight change in conductance parameter values perpendicular to the local curvature of the sheet can yield substantial changes in activity.

A proposed alternative method for modeling a neuron’s activity is to subject a single neuron to complicated perturbations which cause neurons to reveal their individual character and produce sufficiently information-rich responses to recover the underlying conductance parameter values (Hobbs and Hooper, 2008). This recovery is done by searching through the parameter space of a suitable neuron model until a set of conductance parameter values is found which causes the model to behave similarly to the real neuron, as assessed by a suitable distance metric. This process is called parameter optimization. In the most trivial of models, with very few conductance parameters to search and a short simulation time, it is feasible to exhaustively search the parameter space. With most neuron models however, including the Prinz model, combinatorial explosion makes this sort of exhaustive search impossible, and thus heuristic algorithms – methods such as evolutionary algorithms or simulated annealing which trade optimality or precision for speed – are used for parameter optimization.

The performance of heuristics largely depends upon the nature of the difference landscape, i.e., the relationship of conductance parameter values to distance between the activity of the model and the activity of the real neuron. If the difference landscape has only a single minimum and a smooth gradient towards it, then a simple gradient descent will find it. If, however, the difference landscape is pathological (Figure 22), with many local minima or non-monotonic or poor gradients towards the correct set of conductance parameter values, then the likelihood of finding the global minimum and recovering the correct conductance parameters is decreased.
Gradient descents were performed from three different starting positions (*red, green, blue dots*). From a suitable starting position (*green*), descent will reach the global minimum. Gradient descent from a second position (*red*) will reach the basin of equivalence (deep valley, bottom right) but would stop if a local gradient becomes too low, or the “noise floor” is reached. From a third position (*blue*), descent will reach a local minimum distant from either the basin of equivalence or the global minimum.
The difference landscapes of free-running neuron models appear visibly pathological, with widespread basins of equivalence, and a potentially non-monotonic gradient. This is true whether distance is assessed with $D_W$ (Figure 8) or $D_P$ (Figure 10). The existence of widespread regions of low distance is a problem because optimization is likely to stop once the conductance parameters are within the basin and the model’s output is similar to the real neuron; the inherent noise in electrophysiological recordings would prevent further improvement. A non-monotonic gradient is a concern because these heuristics’ performance – both speed of convergence and ability to escape local minimum – decreases with non-monotonicity.

Model neurons which behave identically under free-running conditions do often show different behavior when stimulated, and metrics which compare output waveforms detect these differences (Figure 23). It is thus reasonable to hope that a suitable perturbation could be found which yields well-behaved difference landscapes, wherein neuron models which behave similarly under free-running conditions would separate, and the relationship of conductance parameter values to distance would become more monotonic. Prior research (Hobbs and Hooper, 2008) evaluated white noise in voltage clamp. The use of white noise as a perturbation is common because of its ability to improve reproducibility of spike timing in spiking neurons (Mainen ZF and Sejnowski TJ, 1995), and it was assumed that white noise would eventually drive the neuron through all possible states. An arbitrary target point in parameter space was selected, and the model generated by that point was perturbed with white noise, to yield a target response waveform. Then, an evolution strategy was used (Materials and Methods – Evolution Strategy) which started with a population of models with random conductance parameters. This algorithm, based upon the principles of evolution, calculated the fitness of each initially random model by comparing its response to the white noise perturbation against the target response waveform. In each generation, the fittest models were kept as “parents”, and each parent produced a single
offspring with parameter values similar its parent. After many generations, the parameters of the models in the population converged to the original conductance parameters. This process was repeated for different randomly chosen targets, with the evolution strategy reliably recovering the target conductance parameters.

Figure 23. Perturbation separates neuron models with similar activity.

Three neuron models with different parameters but similar frequency under free-running conditions (A-C) were stimulated with a current-clamp perturbation (D). In response to the perturbation, the models’ responses diverged, and remained distinct in phase and activity.

Despite this good performance *in silico*, attempts to fit the conductance parameters of the Prinz model to the output of real neurons voltage clamped to white noise did not produce appreciably better results than fitting a passive model. This could be due to inadequacies of the Prinz model if, for example, the dynamics or voltage-dependence of the conductances in the Prinz
model do not correspond to those of the neuron. However, it could also be that a white noise perturbation does not activate the conductances sufficiently for the inward and outward currents to appear over the noise in the recording. Neurons are not linear systems, and white noise may not be efficient at producing the particular driving patterns required to cause channels to open and stay open long enough for appreciable current to flow. Examination of the voltage-dependent currents of a pacemaker model neuron in response to white noise confirmed this suspicion (Figure 24).

**Overview of research**

The goal of this phase of research was to find perturbations which offer better performance than white noise in two related, but separate tasks. The first task, referred to herein as *separation*, is distinguishing model neurons which behave similarly under free-running conditions. A good perturbation would cause models with different conductance parameter values to produce different outputs, even if those models occur within the basin of equivalence in free-running conditions. Ideally, such a perturbation would yield increasing distance between output waveforms with increasingly divergent conductance parameter values. Assuming the results in the model translate to similar results in real neurons, this would permit the perturbation to be used to ascertain whether real neurons were more similar or more different in conductance complement. The second task, *pathfinding*, is recovery of a known target set of conductance parameter values starting from a random set of conductance parameter values and attempting to match the response of the target. Good performance implies a well-behaved difference landscape, with relatively few local minima and a consistent gradient towards the target conductance parameters. It should be noted again that the difference landscape depends both upon the perturbation and upon the reference (target) set of conductance parameter values.
Towards this goal, we designed performance tests appropriate to each task which could compare perturbations, using software described in detail in Materials and Methods. The first test selected two random points in parameter space and calculated the distance between their responses to various perturbations. This was repeated until sufficient data were obtained to

Figure 24. White noise does not appreciably activate voltage-dependent conductances.

A pacemaker model neuron (conductance parameters given in Table 1 C) was voltage clamped to both white noise and a simple square wave (A), and the voltage-dependent ionic currents were monitored (B: $I_{CaT}$; C: $I_{Cas}$; D: $I_A$; E: $I_{KCa}$; F: $I_{Kd}$; G: $I_{Hi}$; H: $I_{So}$). Activation of most currents by white noise was minimal.

Towards this goal, we designed performance tests appropriate to each task which could compare perturbations, using software described in detail in Materials and Methods. The first test selected two random points in parameter space and calculated the distance between their responses to various perturbations. This was repeated until sufficient data were obtained to
statistically assess whether some perturbations yielded better separation (i.e., increased distance via the distance metric), and whether that distance reflected the difference in conductance parameter values. The second test selected a random target point and a random test point, and then, for each perturbation, performed a gradient descent of the test point on the difference landscape. Again, this was repeated until sufficient data were obtained to test whether some perturbations resulted in greater movement of the test point towards the target, or whether this travel was faster. In both of these tests, we also wanted to consider whether or not different perturbations would produce better or worse results in different types of model neurons. Accordingly, we repeated each test with different scenarios, limiting the choice of random points in each scenario to different classes of neurons. All tests were performed using the $D_w$ (waveform distance) metric, as this metric was to be used for further phases of research.

We chose several common perturbations for these evaluations. In addition to these perturbations, we hypothesized that it should be possible to empirically design a perturbation which would yield superior performance on the first task, and possibly the second. The design of this perturbation could be achieved using a suitable heuristic method to adjust a perturbation waveform until the amplitude of a single voltage-dependent current is maximized and the amplitudes of all other currents are minimized. By repeating this optimization process for each conductance in the model, a combined perturbation waveform could be designed which sequentially explores the active conductances of the model. While this may not necessarily yield a well-behaved difference landscape, it should cause models with different conductance parameters to elicit different responses, as the current amplitude in each section should reflect that current’s maximal conductance parameter.
Materials and Methods

Pyloric neuron simulations and distance metrics

The Prinz model of decapod crustacean pyloric neurons was used for model simulations (see Chapter 1 for equations), as described in Chapter 1 - Materials and Methods. This model includes seven voltage-dependent currents and one leak current, and was implemented in a single compartment using custom software written in C. The final perturbation was to be used in real *Panulirus interruptus* pyloric neurons, and these stomatogastric neurons are known to contain a persistent sodium current (Elson and Selverston, 1997). Thus, a suitable $I_{NaP}$ was included in the model in some cases. This combined model was designated the “Prinz+NaP” model. This current was governed by the following equations:

\[
m_\infty(V) = \frac{1}{1 + e^{(V+26.8)/-8.2}}
\]

\[
\tau_m(V) = 19.8 - \frac{10.7}{1 + e^{(V+26.5)/-8.6}}
\]

\[
h_\infty(V) = \frac{1}{1 + e^{(V+48.5)/4.8}}
\]

\[
\tau_h(V) = 666 - \frac{379}{1 + e^{(V+33.6)/-11.7}}
\]

\[
\frac{dm}{dt} = \frac{m_\infty - m}{\tau_m}
\]

\[
\frac{dh}{dt} = \frac{h_\infty - h}{\tau_h}
\]

\[
I_{NaP} = \bar{g}_{NaP} \cdot m^3h \cdot (V - E_{Na})
\]

with $E_{Na} = 50$ mV. A point $p$ in the eight-dimensional parameter space of this neuron model is said to generate a specific model neuron, $M(p)$. When stimulated with a particular perturbation waveform $s$, this model neuron produces a unique response waveform $r$, i.e., $M(p,s) = r$. 
The waveform distance metric ($D_W$) was exclusively used in this section of the research. Waveform distance between two response waveforms $r_1$ and $r_2$ was calculated as:

$$D_w(r_1, r_2) = \frac{1}{d} \int_0^d |r_1(t) - r_2(t)|^p$$

where $d$ is the total duration, and $p = 1$. To prevent the initial conditions of the integrator from affecting model results, and allow for rhythmic activity to begin in free-running conditions, the first 5 s of output were not used in distance measurements. Distance was always measured between response waveforms resulting from models subjected to the same perturbation. Accordingly, for notational brevity, the waveform distance between two response waveforms $r_1$ and $r_2$ resulting from models $M(p_1, s)$ and $M(p_2, s)$ is referred to as $D_w(s|p_1, p_2)$.

**High-performance computing**

Where possible, algorithms were parallelized to improve performance, using an in-house high-performance computing cluster of 8 hosts running Fedora Linux, 8 cores per host, 2 threads per core. OpenMPI was used as the communications layer with additional infrastructure software written in Perl. Unless otherwise specified, all software was custom written in C.

**Parameter-space raster scans**

The rasterscan program, described in *Chapter 1 – Materials and Methods*, was used to perform regular grid searches (raster scans) of parameter space slices around a reference point $p_{\text{ref}}$ using a given set of perturbations $s_1, s_2, ..., s_n$. The values of two out of the eight conductance parameters were iterated across a plane at 256 regular intervals, for a total of $256^2$ points. All other conductance parameter values were held constant and equal to the corresponding values in $p_{\text{ref}}$. At each raster-scanned point $p$, waveform distance $D_w(s|p, p_{\text{ref}})$ was calculated and recorded for each perturbation $s = s_1, s_2, ..., s_n$. The data were visualized as surfaces, with the $x$ and $y$
coordinates corresponding to the conductance parameters being varied, and $z$ corresponding to waveform distance.

**Evolution Strategy**

A subtype of evolutionary algorithms, an *evolution strategy*, was used to construct a perturbation which maximized the activity of a voltage-dependent current against the background of all other currents. An evolutionary algorithm utilizes the principles of evolution; evolution strategies are those evolutionary algorithms which specifically optimize real-valued parameters.

The evolution strategy was largely based upon standard methods (Fogel, 1994; Yao X et al., 1999), with non-standard extensions to improve performance and avoid local minima. The standard algorithm begins with a population of individuals each with a different random *genotype*, in this case being the perturbation waveforms. At each generation, the individuals compete against each other based upon *fitness*, in this case the degree to which the waveform activates a desired ionic current in a model neuron. The winners, those waveforms which elicit a greater amount of the desired current, are kept as parents, and the losers are discarded. The parents then produce offspring, with each parent producing an initially identical child whose parameters are then mutated. Pairs of individuals are then selected for recombination, during which sections of parameters are exchanged between individuals. The new population then begins another round of competition, and the process continues, with the population gradually improving in fitness and, ideally, converging to a desirable set of parameters.

The initial evolution strategy algorithm gave poor performance at evolving a perturbation waveform for the voltage-dependent currents with inactivation gates, with only moderate progress occurring after thousands of generations. To improve performance, the concept of *genetic drift* was added by choosing a small number of individuals to compete, mutate, and recombine in isolated *colonies*. Genetic drift was initiated at the beginning of the evolutionary process by
starting with several isolated colonies, and new colonies were created whenever overall improvement in fitness had stagnated. After a number of generations wherein these individuals could evolve separately, they were then rejoined with the main colony. This permitted desirable waveform motifs to evolve in isolation and be joined together through recombination. The inclusion of genetic drift considerably increased the performance of the evolution strategy, yielding higher fitness perturbations and producing results in fewer generations.

A second performance enhancement was to change the tuning parameters when stagnation occurred. Many of the steps in the evolution strategy involved parameters which governed factors such as whether individuals competed against all others or only a subset of the population, or how frequently recombination occurred. It was observed that a particular set of values for these tuning parameters would yield good performance at some stages of the evolutionary process but poor performance at others. Accordingly, when colony fitness failed to improve, these tuning parameters were randomly changed. As with genetic drift, this process increased performance and yielded better results.

Finally, a third performance enhancement was to do the initial evolutionary process using relatively few ($n = 128$) waveform parameters describing the waveform being evolved. After a suitable number of generations had passed, and short waveforms had evolved, each waveform was doubled in size, by appending a copy to itself; this doubled both the number of samples in the waveform, and the total duration. As many of the conductance-optimized waveforms were periodic, this enabled the evolution strategy to evolve the initial motifs more rapidly, and then fine tune them after they were repeated. After a suitable number of additional generations, a second doubling step was performed, to construct the final waveform characterized by 512 waveform parameters.
Due to the complexity of the algorithm, and in particular the performance optimizations, the algorithm is described conceptually below. Full details are made available in Appendix 1: Software implementation details.

Definitions and notation

In the evolution strategy, an individual is a data structure which contains several different components. Each individual is assigned a number, designated $i$. These components include:

1. The individual’s genotype, a set of real-valued waveform parameters to be optimized, described below. In this implementation, all individuals had the same number of waveform parameters.

2. The mutation sizes, one corresponding to each waveform parameter. These are used as standard deviations for the mutation step and thus govern the degree of mutation.

3. The colony number, designated $\zeta_i$, which specifies to which colony the individual belongs.

4. The drift counter, designated $\beta_i$, specifying how many more generations remain before the individual is placed into the main colony (colony 1).

5. The fitness value, $\phi_i$, a real number giving the fitness score associated with the waveform parameters. In this implementation, lower fitness scores were more desirable. The fitness function is described below. The fitness value of a newly constructed individual is undefined.

Note that the colony number and drift counter are non-standard components.

The population is the collection of individuals participating in evolution, and includes a fixed number of individuals designated parents, and an equal number of individuals designated children. In this implementation there were 2000 parents and 2000 children, excepting the first generation which had 4000 parents and no children.
Genotype

The genotype of an individual was the set of waveform parameters corresponding to a waveform comprising connected ramp segments (Figure 25). Each pair of waveform parameters represented the amplitude at the end of a ramp segment and its duration. The amplitudes at the beginning and end of the first ramp were identical, allowing the algorithm to begin the waveform with a lead-in holding potential. Amplitudes were constrained between -5 mV and -100 mV, and durations between 0.05 ms and 50 ms. Each ramp was further limited to a maximum $dV/dt$ of 10 mV/ms, to prevent damaging currents or ringing if used in voltage clamping real neurons.

![Figure 25. Example ramp waveform and waveform parameters.](image)

Parameters for first several ramps are labeled (red: voltage parameters; blue: duration parameters).

Fitness function

The fitness function was designed to provide evolutionary pressure to maximize the amount of the desired voltage-dependent current appearing against the background. The waveform to be evaluated was provided as a perturbation to a Prinz+NaP model neuron whose $g$
values were all arbitrarily set to 100 mS/cm$^2$. While this does not match the conductance complement of any known neuron, it was hypothesized that a voltage clamp perturbation which was optimized to elicit a given current against the background of all others would still be good at doing so in any neuron regardless of its actual conductance parameters. Integration began with an initial condition of all gating particles being at steady state at the voltage clamp potential of the first (flat) ramp. During integration, at each time step, the values of each voltage-dependent current and capacitive current ($C \cdot dV/dt$) were monitored. These were joined to create current amplitude waveforms for each such current (as in Figure 24). At each time $t$ (sampled at 1 ms intervals), a reward score $v_r(t)$ and a penalty score $v_p(t)$ were calculated according to the following general rules (details given in Appendix 1).

- The reward score $v_r(t)$ was proportional to the amplitude of the desired current.
- The penalty score $v_p(t)$ was proportional to the sum of all other currents, including the capacitive current.
- The penalty score $v_p(t)$ was scaled according to the magnitude of the desired current.

The final fitness score was calculated as $\int (v_r(t) - v_p(t)) \, dt$.

Scaling the penalty by the magnitude of the desired current avoided penalizing for sections of waveform where the desired current was absent but other currents were present. This was particularly important when the desired current was inactivating, as it facilitated evolution of rhythmic waveforms with alternating activation and rest sections. During activation sections, the target current was elicited; these sections continued until the target current began to inactivate or other currents began to compete. During rest sections, inactivation of the target current was removed, and other competing currents could be active without penalty.
Initialization

New parent individuals were created at the beginning of the first generation. Each was initialized by setting its waveform parameters to random values chosen from a flat distribution with suitable bounds (for amplitudes, -80 mV to -20 mV; for ramp durations, 5 ms to 20 ms), to construct a random waveform. The mutation sizes were set to 2% of the corresponding waveform parameter’s range (i.e., the mutation size was 1.2 mV for amplitude parameters, and 0.3 ms for duration parameters). Individuals were assigned round-robin to one of 16 different colonies, and each individual was assigned a normally distributed random drift counter value (μ = σ = 400 generations), so that they would gradually merge back into the main colony.

Fitness scoring

Each generation started with fitness scoring in any individuals with undefined fitness scores. In the first generation this included all parents; in subsequent generations, all children.

Selection

Individuals competed only within a colony. Every individual, both parents and children, competed, against some or all of the other individuals within the colony. A round of competition consisted of comparing fitness scores; the winner was the individual with the better fitness. At the end of all rounds of competition, the individuals were ranked according to the number of wins. The top half became the new parents, and the bottom half were eliminated.

Mutation

During mutation, each parent spawned one child within the same colony. Each of the child’s waveform parameters was then mutated, by adding a random number from a Cauchy distribution multiplied by the mutation size. The child’s mutation sizes were themselves mutated as the algorithm proceeded, decreasing them by a random amount using a standard exponential formula. In this way, the mutation size gradually decreased as the population evolved. The
Cauchy distribution was used instead of a normal distribution to increase the frequency of larger changes.

Recombination

Some of the children within each colony were selected for recombination. Each child was either recombined with a randomly selected child (changing both children’s genotypes), or their genotype was entirely replaced with the recombination of two parents (the parents were unaffected by the operation). Recombination occurred by choosing random crossover points and exchanging portions of the genotype, thus creating a chimeric waveform made of alternating segments from the two original waveforms.

Genetic drift

In the final step in a generation, each individual’s drift counter was decremented, and if it reached 0, the individual was merged back into the main colony. Then, the entire population was assessed for stagnation. Should no appreciable improvement in fitness have occurred within the colony within ten generations, one of several steps was chosen randomly:

- Some individuals from the main colony were assigned to a new drift colony to start a new phase of genetic drift. These seed individuals’ mutation sizes were increased to their original values, and in some cases their waveform parameters were mutated. This prevented the new colony from converging to the same waveform parameters as the main colony, and instead allowed the new colony to find its own high fitness waveforms.
- Some individuals were reinitialized with the original waveform parameter distribution and mutation sizes.
- The various tuning parameters affecting mutation, recombination, and genetic drift in the entire population were randomly altered (see Appendix 1 for bounds).
Conductance-Optimized Perturbations

The evolution strategy was used to design voltage clamp stimulus waveforms for the eight voltage-dependent conductances of the Prinz+NaP model. One individual perturbation segment was created for each of the eight voltage-dependent conductances in the Prinz+NaP model. For each segment, the population began with a population of 4,000 random waveforms comprising 64 voltage ramps described by 128 waveform parameters (Figure 25), and evolved this population through 20,000 generations, with the number of waveform parameters doubling at the 4,000th and 8,000th generations. The eight final segments were combined into a single composite perturbation, designated opt, by concatenating them together, with 5 s intervals between segments during which the voltage was set to the same value as the beginning of the next segment. This was done to allow the neuron to reach a steady state condition prior to the beginning of a segment.

Current clamp waveforms were also evolved using the same evolution strategy. For the current clamp waveforms, clamp amplitudes were constrained between -20 nA and 20 nA. Unlike voltage clamp, it was hypothesized that the model neuron’s conductance parameters would substantially affect the optimization process, because of the feedback loop between changes in membrane voltage, and changes in the states of the voltage-dependent conductances. Such a feedback loop does not occur in voltage clamp, because membrane voltage is constrained. In current clamp, therefore, the waveforms appropriate to a model neuron with a given set of conductance parameters might be unsuitable for a neuron with a different set of conductance parameters. We chose the conductance parameters of a previously published pacemaker neuron (Na: 20; CaT: 2.5; CaS: 2; A: 40; KCa: 5; Kd: 125; H: 0.01; all values µS/cm²) and added a small persistent sodium current (10 µS/cm²) and a small leak current (0.1 µS/cm²), under the
assumption that this might result in suitable perturbations for rhythmic neurons. The current clamp perturbations were joined together similarly.

*Other perturbations*

Several voltage clamp and current clamp perturbations were used throughout this research. All perturbations were initially made 60 s long. When compared with the composite optimized perturbations in the separation and pathfinding performance tests, these were extended to 200 s (voltage camp) and 184 s (current clamp) by repeating them until the desired duration. These perturbations included the following.

*White noise*

White noise (a random signal with a spectrum where power is inversely proportional to frequency) (*Figure 26A*) was constructed by sequentially generating random voltages between -100mV and -5 mV, at 1 ms intervals. These were then low-pass filtered at 20 Hz with an FFT corner filter, i.e., performing a fast-Fourier transform, removing all frequency components above 20 Hz, and then computing the inverse transform. As this reduced the amplitude of the waveform substantially, it was then rescaled and offset in voltage until 95% of its voltage values were again within the desired -100 mV to -5 mV range. It was then clipped to this range. Scaling to constrain 95% followed by clipping yielded a waveform with substantially more excursions towards the depolarized and hyperpolarized extremes than simply scaling the entire waveform within -100 to -5 mV. A white noise current clamp waveform was similarly constructed, ranging from -0.2 nA to 1 nA.

*Pink noise*

Pink noise (a random signal with a power spectrum) (*Figure 26B*) was generated similarly to the white noise waveform. Instead of an FFT corner filter, an envelope filter was used; the complex amplitude at a given frequency $f$ was scaled by $1/f^{0.5}$; amplitudes above $f = 20$
Hz were set to 0. The pink noise waveform was scaled similarly to the white noise waveform to range within -100 to -5 mV. A pink noise current clamp waveform was similarly generated, ranging within -0.2 to 10 nA.

Figure 26. White and pink noise perturbations.
Five seconds of white (A) and pink noise (B) voltage clamp perturbations shown.

Chirp

A chirp (sine wave whose frequency increased with time) (Figure 27A) was generated with the following equation:

\[ v(t) = \sin\left(2\pi \cdot t \cdot e^{\varphi(t)}\right) \]

\[ \varphi(t) = \log(f_{min}) + (\log(f_{max}) - \log(f_{min})) \cdot \frac{t}{t_{max}} \]

with \( f_{min} = 0.01 \) Hz, and \( f_{max} = 20 \) Hz. This produced a chirp with a logarithmically increasing frequency. This was then clipped between 0.8 and -0.8, to extend the duration at hyperpolarized and depolarized extremes, and then scaled and offset to range between -5 and -100 mV to produce the voltage clamp chirp perturbation. A current clamp chirp perturbation was constructed similarly, initially ranging between -1 nA and 1 nA; all negative values were then multiplied by 0.2, to constrain the range within -0.2 nA to 1 nA.
Randomized chirp (rchirp)

A randomized chirp waveform (a random noise waveform whose bandwidth increased with time), designated rchirp (Figure 27B), was created by starting with white noise, and filtering it in the time/frequency domain with a low pass filter whose cutoff frequency increased with time. This was done using a Gabor transform (Qian S and Chen D, 1993). To compute the Gabor transform of a waveform, the waveform is multiplied by a Gaussian function, and then a Fourier transform is performed on the result. The Gaussian function, the window, is then offset in time, and the process is repeated. Mathematically, the Gabor transform is defined as:

$$G_x(t, f) = \int_{-\infty}^{\infty} e^{-\pi(\tau-t)^2} e^{-2\pi i ft} x(\tau) \, d\tau$$

In practice, the integral is computed between fixed bounds (-1.9 to 1.9 is typical), as the Gaussian is small outside these limits. The inverse transform is

$$x(t) = \int_{-\infty}^{\infty} G_x(t, f) e^{2\pi i ft} \, df$$

The initial white noise waveform was Gabor transformed, sampling at $M = 600$ time and $N = 600$ frequency values. This was filtered:

$$G_x'(m, n) = G_x(m, n) \cdot \Phi\left(\frac{m-1}{M}, \frac{n-1}{N}\right)$$

where $m = 1 .. M$, $n = 1 .. N$, and

$$\Phi(a, b) = \begin{cases} 
1 & \text{if } b \leq 0.04a^{3.5} \\
1.3a^{3.5} + 0.2 & \text{if } b > 0.04a^{3.5}
\end{cases}$$

As time increases, the filter function diminishes in magnitude, and its frequency cutoff increases (Figure 28). The filtered Gabor-transformed white noise was then reverse transformed to produce a random waveform whose bandwidth increased in time. This waveform was scaled.
until 95% of all voltages were within -100 to -5 mV and then clipped to this range. The Gabor transform was implemented in Octave using the \texttt{ltfat} module version 0.96.

A noisy chirp was generated by taking chirp and white noise waveforms, and combining them proportionally, beginning with 100% chirp, ending with 50% chirp and 50% white noise (\textit{Figure 27C}): 

\[ \text{chirp}(t) = \text{chirp}(t) + 0.5 \frac{t}{t_{\text{max}}} (\text{white}(t) - \text{chirp}(t)) \]
This perturbation was used only for current clamp, as it was discovered in initial testing to produce poor results in voltage clamp.

![Composite optimized perturbations](Image)

**Figure 28.** Profile of time-frequency domain filter used to create randomized chirp.

This low-pass filter with increasing bandwidth with time was applied white noise to produce a randomized chirp, using a Gabor transformation. At the start of the perturbation, the cutoff frequency was low, with the gain high. As time increased, the cutoff frequency increased, eventually reaching 20 Hz. Gain decreased as bandwidth increased, maintaining constant power in the filtered waveform.

**Phase-locking pulse**

A phase locking pulse perturbation was used in current clamp to produce free-running activity with predictable phase. This perturbation consisted of an initial 100 ms of no stimulation, followed by 100 ms at -0.2 nA, then 800 ms at 1 nA, returning to 0 nA.

**Composite optimized perturbations**

Three composite voltage clamp perturbations were constructed (*Figure 29*) which included the evolution strategy-optimized perturbation *opt* conductance-optimized perturbation.
For voltage clamp, the \textbf{cp1} (composite perturbation 1) perturbation consisted of an \textbf{opt} section, 40 s pink noise, a second \textbf{opt} repeat, and a 40 s \textbf{chirp}. The \textbf{cp2} perturbation consisted of two \textbf{opt} repeats, a 40 s \textbf{chirp}, two more \textbf{opt} repeats, and 40 s pink noise; in this perturbation, the 5 s holding periods between segments in the \textbf{opt} perturbation were replaced by 0.5 s holding periods. Finally, the \textbf{ropt} (repeated \textbf{opt}) perturbation was simply 5 \textbf{opt} repeats with 0.5 s inter-segment holding periods. All three composite voltage clamp perturbations were 200 s long. Two composite current clamp perturbations were also constructed, both 184 s long. The \textbf{cp1-cc} current clamp perturbation consisted of an \textbf{opt} section, a \textbf{rchirp} section of 40 s, a second \textbf{opt} repeat, and an \textbf{nchirp} section. The \textbf{ropt-cc} current clamp protocol consisted of two \textbf{opt} repeats.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{composite_voltage_clamp_perturbations.cp1.cp2.ropt}
\caption{Composite voltage clamp perturbations \textbf{cp1}, \textbf{cp2}, and \textbf{ropt}}
\end{figure}
Test scenarios

Initially, it was suspected that perturbation effectiveness might vary based upon the type of neuron; for example, a perturbation good for separation or pathfinding with a rhythmically active model neuron might not be ideal for a quiescent neuron. Accordingly, five different scenarios were developed, governing the selection criteria used to pick pairs of model neurons to compare against each other. In each scenario, the choice of conductance parameter values used to construct model neurons was restricted in different ways. In some scenarios, the fast sodium (“-Na” scenarios) or leak (“-L” scenarios) were removed from the models, and pathfinding was restricted to the remaining conductance parameters. These scenarios included the following.

Scenario Iso-Na-L: isochronous neurons from basins of equivalence

Previous research (Chapter 2) had identified a large pool of model neurons which were closely isochronous to a bursting pacemaker model (i.e., within its basin of equivalence), low in waveform distance from that neuron, and generally from each other. A principle goal of this research was determining whether perturbations could separate neurons which behaved similarly under free-running conditions, or allow recovery of conductance parameters in neurons within a basin of equivalence. Parameter space points within the $D_w$ basins of equivalence for the reference oscillating model neuron were gathered; sources included all raster scans (Chapter 2 – Results - Raster scan surfaces and volumes), and all flood fill data (Chapter 2 – Results - High-resolution flood fill explorations). Neither fast sodium nor leak conductances were present in any of these model neurons, and these conductances were disabled during separation and pathfinding tests. These model neurons all had low waveform distance to the reference model, but not necessarily to each other. To increase the likelihood that they would have low waveform distance to each other, and thus be closely isochronous, only those points whose waveform distance was within $\varepsilon = 2$ of the reference model were used. These were assembled into a pool of 20,000
neurons from which pairs of model neurons were chosen randomly with replacement. This scenario was designated Iso-Na-L (isochronous models, without fast sodium or leak).

_Scenarios PvR-L and PvR-Na-L: pacemaker vs. randomly chosen_

A second goal was to assess whether different perturbations could distinguish rhythmically active pacemaker neurons from arbitrary other neurons, and assess how differently perturbations might affect pathfinding with pacemaker neuron targets. In the _PvR-L_ (pacemaker vs. random, without leak) scenario, one model neuron of the pair was chosen randomly with replacement from a small set of previously published pacemaker neurons (all listed in _Table 6_) (Prinz et al., 2003). These neurons were qualitatively similar – all produced rhythmic bursting activity – but had substantially different frequencies and waveform shapes. The other model neuron was chosen randomly in parameter space, but with no leak conductance, as this was absent from all of the pacemakers. In the _PvR-Na-L_ scenario, the first model neuron of the pair was chosen from the same pool of bursting model neurons but its leak and fast sodium conductances were eliminated, converting it from a burster to a slow-wave oscillator. The second was chosen randomly in parameter space, without fast sodium or leak present. When assessing pathfinding performance, the first neuron in the pair, chosen randomly from the small set of pacemakers, was used as the target neuron, and the second, chosen randomly in parameter space, was used as the “raindrop”.

Table 6

Pacemaker Neuron Pool for PvR Scenarios

<table>
<thead>
<tr>
<th>CaT</th>
<th>CaS</th>
<th>A</th>
<th>KCa</th>
<th>Kd</th>
<th>H</th>
<th>Na</th>
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<td>40</td>
<td>5</td>
<td>125</td>
<td>0.01</td>
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<td>10</td>
<td>5</td>
<td>125</td>
<td>0.01</td>
<td>300</td>
</tr>
</tbody>
</table>

Values are maximal conductances for specified currents, in mS/cm². For the “-Na” scenarios, no sodium conductance was present. Leak conductance was not used in these scenarios.

Scenarios RvR and RvR-Na-L: random vs. random

The final set of scenarios considered pairs of neurons chosen randomly within parameter space. These generated model neurons which could be quiescent, tonic spiking, oscillating, tonic depolarized, or irregular in activity. These scenarios were intended to test how well different perturbations performed in the general case. In the RvR scenario, both the first and the second model neurons were chosen randomly in parameter space. In the RvR-Na-L scenario, both model neurons were chosen randomly, with leak and fast sodium conductances were disabled.
Assessing separation performance

The effect of perturbation on separation performance was characterized with the pair_errors program, custom software written in C. This software assessed how perturbation choice affected waveform distance $D_W(s|p_1, p_2)$ measured between pairs of model neurons chosen according to selection scenario (Iso-Na-L, PvR-Na-L, etc). A set of $n$ perturbation waveforms, $s_1, s_2, \ldots, s_n$ was loaded into memory. Two parameter space points, $p_1$ and $p_2$ were chosen according to the selection scenario. $D_W(s|p_1, p_2)$ was then calculated for all $s$ in $(s_1, s_2, \ldots, s_n)$. A new pair was selected, and waveform distances calculated, repeating for the desired number of iterations.

Assessing pathfinding performance

The effect of perturbation choice on pathfinding (recovery of original conductance parameters by gradient descent) performance was characterized with the raindrop program, custom software written in C. This method was used instead of a heuristic algorithm such as an evolution strategy due to the large computation demands of these algorithms and the need to identify local minima in the difference landscapes as one of the criteria for ranking perturbations. Conceptually, this program can be understood as placing “raindrops” onto a difference landscape, letting them flow downhill until reaching a local minimum, and then measuring how closely they approached the target and how quickly they moved (Figure 30). If the raindrop reaches the target, this indicates a lack of local minima along the path of descent; if it moves quickly, this indicates a steep gradient. A set of stimulus waveforms, $s_1, s_2, \ldots, s_n$ was loaded into memory. A target conductance parameter vector $p_{\text{target}}$ and a random “raindrop” point $p_{\text{drop}}$ were selected according to scenario. A gradient descent was then performed, moving the raindrop, using the Nelder-Mead simplex (“amoeba”) method (Nelder JA and Mead R, 1965) for the first perturbation $s_1$, described below. The gradient descent stopped when either a local minimum was
reached, or when the simplex step size had contracted to $1 \times 10^{-4}$ its original value. The gradient descent was then repeated for every remaining perturbation, using the same target and initial drop points. For each perturbation, the program recorded the final position reached by the simplex, and the number of iterations required to reach it. A new target and drop were chosen, and the process was repeated.

The Nelder-Mead simplex method began by selecting a simplex around $p_{\text{drop}}$. A simplex is a polytope of $n+1$ points, with $n$ being the number of dimensions of the parameter space (either 8, 7, or 6, depending on whether leak and/or sodium conductances were enabled). For example, a simplex in two dimensions is a triangle; a simplex in three dimensions is a tetrahedron. Then, the waveform distance $D_W(s|p_{\text{target}}, p_i)$, for each simplex point $p_i$ was calculated. These were ordered by waveform distance, and the simplex point with the greatest distance was discarded. A centroid was calculated by taking the mean of all remaining points, and a new $n+1$th vertex was calculated by reflecting the discarded point across the center. If the new vertex generated a model whose waveform distance from the target was lower than the remaining simplex points, this indicated a successful downhill movement, and the reflection distance was increased. If the new vertex’s waveform distance from the target was not lower, the reflection distance was decreased, as this potentially indicated a leap across a valley. Gradient descent continued until a local minimum was reached, or until the simplex had contracted to a size below threshold. Upon exit from the gradient descent, the simplex point with the lowest waveform distance was returned.
Figure 30. Pathfinding assessment with the raindrop program.

A target point and raindrop point in parameter space were chosen according to selection scenario, e.g., isochronous non-spiking model neurons in the Iso-Na-L scenario. Red, green, and blue dots on the surface show descents from three different starting positions (note that the raindrop program chose a new target with each new raindrop). A gradient descent was performed using the Nelder-Mead “amoeba” method on the difference landscape generated by the target. Descent stopped when a local minimum was reached, or when the simplex had contracted below a threshold, indicating a shallow gradient. Conductance parameter distance between the final raindrop location (red, green, or blue dots on xy plane) and target (black dot) was then recorded.
Statistical analysis

For **pair_errors** data, for each scenario, an ANOVA was conducted on the corresponding $D_W$ values over the different perturbation protocols. When it was found that $D_W$ varied by perturbation, a Tukey's Honest Significant Differences post-hoc test was used to determine whether specific perturbations produced greater $D_W$ values than others. For each scenario, linear models were then made for each perturbation, to attempt to fit the $D_W$ values to the differences in conductance parameter values, i.e.,

$$D_W(p_1, p_2) = \beta_0 + \beta_{Cat}(|p_1_{Cat} - p_2_{Cat}|) + \beta_{Caa}(|p_1_{Caa} - p_2_{Caa}|) + \cdots + \epsilon_{p_1, p_2}$$

An approximate linearity rank was assigned to each perturbation within a given scenario, by sorting first on the number of conductance parameters successfully included in the linear model, and second by the F score of the linear model. A conductance parameter was considered to be “successfully fit” if its presence in the linear model resulted in significant improvement by F test, and if its coefficient was $> 0$ (i.e., positive correlation).

For **raindrop** data, again for each scenario, a progress value $\varphi$ was assigned for each individual conductance parameter as $(d_{init} - d_{final}) / d_{init}$ where $d_{init}$ was the initial absolute difference between the target and test conductance parameters, and $d_{final}$ was the absolute distance between target and test conductance parameters after the gradient descent. For example, if the target had $\overline{g_A} = 40$, and the raindrop began with $\overline{g_A} = 10$ and ended with $\overline{g_A} = 30$, then $\varphi_A = (|40 - 10| - |40 - 30|) / |40 - 10| = 0.67$. When distance increased rather than decreasing, $\varphi = 0$ was assigned. Thus, for each conductance parameter, then $\varphi$ ranged from 0 (no progress, or increasing distance) to 1 (when the conductance parameter reached the same value as the target). A combined progress $\varphi_{tot}$ was calculated as the Euclidean distance of the conductance parameter progress values, $\sqrt{\varphi_{Cat}^2 + \varphi_{Caa}^2 + \cdots}$. A separate speed value, $s$, was assigned for each
conductance parameter as \((d_{\text{init}} - d_{\text{final}}) / \text{steps}\), and a combined speed value was derived similarly as the Euclidean distance of all conductance parameter speed values.

ANOVA and, if appropriate, Tukey's HSD post-hoc tests, were then conducted to determine whether some perturbations produced significantly greater progress, or faster speed, with respect to a given conductance parameter or to the combined scores. Perturbations were ranked according to the number of significant differences in all progress post-hoc results, and again in all speed post-hoc results.

Where the same data were used to test multiple hypotheses, e.g., when assessing the impact of perturbation choice on progress for the different conductance parameters, the Holm-Bonferroni correction method was used to calculate adjusted p-values. This correction is performed as follows. Given \(n\) different tests conducted on the same data, the \(n\) different p-values \(p_1, p_2, \ldots, p_n\) are ranked in increasing order, e.g., 0.001, 0.005, 0.03, 0.04. Each \(p_i\) is then adjusted to \((n-i+1)\*p_i\); as soon as an adjusted p-value exceeds 0.05, this and all subsequent tests are considered to not be significant. For example: 0.001*4 < 0.05 (significant); 0.005*3 < 0.05 (significant); 0.03*2 > 0.05/2 (not significant). When such p-values were reported, these were the adjusted values.

Statistical analyses were performed with the public domain statistical computing language R (r-project.org) using ANOVA (using the \texttt{aov} module), Tukey's Honest Significant Difference post-hoc test (\texttt{TukeyHSD}), and linear regression models (\texttt{lm}). Post-hoc tests were not conducted when ANOVA results were not significant. Results from voltage and current clamp were always considered separately, as were results from different scenarios.
Results

Conductance-optimized perturbations

The evolution strategy (Materials and Methods - Evolution Strategy) was used to automatically design perturbation waveforms to maximize specific voltage-dependent currents against the background of other currents (Materials and Methods - Conductance-Optimized Perturbations). Once the evolution strategy was performance-tuned, evolution was rapid, both in clock time and number of generations. The initially random perturbations typically became visibly structured within 10-20 generations, with increasing amounts of the desired current appearing (Figure 31 and Figure 32). The speed of evolution was highly dependent upon the performance tuning; the seemingly arbitrary constants in the fitness function, recombination parameters, etc. (Appendix 1) reflect the endpoint of this tuning. The inclusion of genetic drift in the evolution strategy algorithm in particular substantially improved both evolution speed and avoidance of local minima, i.e., sub-optimal perturbation waveforms.

Conductance-optimized perturbations were created for each voltage-dependent current, $I_{CaS}$, $I_{CaT}$, $I_A$, $I_{KCa}$, $I_{Kd}$, $I_{H}$, $I_{Na}$, and $I_{NaP}$. The perturbations optimized to elicit $I_H$ and $I_{KCa}$, both non-inactivating currents, were trivial, consisting of a long continuous stretch of hyperpolarized and depolarized potential, respectively, the other currents being small or zero due to inactivation. Typical motifs for the remaining currents, such as $I_A$ and $I_{Kd}$, included periods of hyperpolarized holding potentials, to remove inactivation of the current, alternating with depolarized segments activating the current. The specific timing and potentials differed from one target current to another, based upon the voltage and time dependence of their activation and inactivation processes. Residual randomness was observed in regions where there was little evolutionary pressure to remove it, such as the hyperpolarized regions in the $I_A$ stimulus, where any reasonably hyperpolarized potential was sufficient to remove inactivation. The eight conduction-optimized
perturbation segments were then joined together sequentially in time to make the **opt** voltage clamp perturbation (*Figure 33*). Each individual section was preceded by a 5 s holding period at the potential of the first ramp in the section, to allow the gates to reach their steady-state value (the conditions under which the perturbations evolved).

The **opt** voltage clamp perturbation was then joined with two other non-optimized perturbations which performed well in preliminary tests to create the **cp1** and **cp2** perturbations, and repeated five times to create the **ropt** perturbation (*Materials and Methods - Other perturbations - Composite optimized perturbations*). These three were all 200 s duration; accordingly, the non-optimized perturbations were extended to this duration to make them comparable in testing. By a similar process, a hypothetically optimized set of current clamp perturbations were generated. These segments were joined with 5s rest periods of 0 nA stimulation to make the **opt** current clamp protocol (*Figure 34*), and used to build two composite perturbations, **cp1-cc** and **ropt-cc**, both 184 s.
**Figure 31.** Evolution of perturbation optimized for $I_A$.

The top ranked perturbation waveform is shown after 5 (A), 20 (C), and 100 (E) generations. Structure evolved rapidly. The responses of the voltage-dependent currents to the evolving perturbations are shown on the left (B, D, F). Amplitude of the target current, $I_A$, is shown in black, along with the absolute value of undesired currents (red: $I_{CaT} + I_{CaS}$; green: $I_{KCa} + I_{Kd}$; blue: $I_H$).
**Figure 32.** Evolution of perturbation optimized for $I_{Kd}$

The top ranked perturbation waveform is shown after 5 (A), 20 (C), and 100 (E) generations. The responses of the voltage-dependent currents to the evolving perturbations are shown on the left (B, D, F). Amplitude of the target current, $I_{Kd}$, is shown in black, along with the absolute value of undesired currents (red: $I_{CaT} + I_{CaS}$; green: $I_{KCa} + I_A$; blue: $I_H$).
Figure 33. Voltage clamp optimized perturbation.

A: Combined perturbation, containing $I_{\text{KCa}}$, $I_{\text{Na}}$, $I_{\text{Kd}}$, $I_{\text{NaP}}$, $I_{\text{CaS}}$, $I_{\text{CaT}}$, $I_{\text{H}}$, and $I_{\text{A}}$ sections. B-G: 1 s samples of the perturbations optimized to elicit $I_{\text{Na}}$ (B), $I_{\text{Kd}}$ (C), $I_{\text{NaP}}$ (D), $I_{\text{CaS}}$ (E), $I_{\text{CaT}}$ (F), and $I_{\text{A}}$ (G). $I_{\text{KCa}}$ and $I_{\text{H}}$ perturbations are largely trivial (not shown), the former (around 8 s in panel A) consisting of a section of depolarized potentials, the latter (around 55 s in panel A) a section of hyperpolarized potentials.
Figure 34. Current clamp optimized perturbation.

A: Combined perturbation, containing $I_{CaS}$, $I_{CaT}$, $I_A$, $I_{KCa}$, $I_{Kd}$, $I_H$, $I_{Na}$, and $I_{NaP}$ sections. B-G: 1 s samples of the perturbations optimized to elicit $I_{CaS}$ (B), $I_{CaT}$ (C), $I_A$ (D), $I_{KCa}$ (E), $I_{Kd}$ (F), $I_H$ (G), $I_{Na}$ (H), and $I_{NaP}$ (I).
Voltage clamp separation performance results

Seven 200 s voltage clamp perturbations were compared using the pair_errors program in the five different selection scenarios (comparing isochronous model neurons without sodium or leak, pacemakers vs. random neurons without leak and with or without sodium, and random vs. random with or without sodium and leak). All data were gathered and analyzed separately for each scenario. Within each scenario, mean waveform distance varied considerably by perturbation, though standard deviations were generally high (Figure 35). White noise, pink noise, and rchirp were poor performers in all scenarios. An analysis of variance (ANOVA) with Tukey’s HSD post-hoc test conducted separately on each scenario established that \( D_W \) differed significantly with different perturbations, and that some perturbations were indeed better than others at eliciting waveform differences, with the composite conductance-optimized perturbations ropt, chirp, and cp2 all performing especially well. Ranking these by the number of significant differences above all other perturbations yielded similar results in each scenario, with ropt consistently outperforming other perturbations (Table 7).

Two dimensional parameter space slices were then explored using the rasterscan program, to calculate \( D_W(s,p_{ref},p_x) \) for the different perturbations, varying \( p_x \) in a regular grid search. This was repeated for various reference models. The ability of different perturbations to produce different waveform distances was reflected in distinctions between the difference landscapes (Figure 36). The composite optimized perturbations yielded difference landscapes which were visibly steeper than the difference landscapes of the other perturbations. For some perturbations, waveform distance did not respond to changes in some conductance parameters; the white noise difference landscape in particular was notably flat in some dimensions. This suggested that some perturbations may be incapable of activating some voltage-dependent
currents to a sufficient degree for changes in the maximal conductance values of those currents to affect the output waveform.

To determine whether this could be the case, linear regression models were constructed for each perturbation to correlate waveform distance with the differences in conductance parameter values (Materials and Methods – Statistical analysis). The β coefficients, intercepts, and residual errors are shown in Figure 37. Again, this was done separately for each scenario. In the Iso-Na-L scenario (isochronous models taken from a basin of equivalence), the residual error of the linear models was relatively small regardless of perturbation; in all other scenarios, residual error was considerably larger. For all scenarios, the linear models made for most perturbations included almost all of the conductance parameters, i.e., waveform distance dependent significantly upon most of the conductance parameters. Ih was a notable exception; linear models did not include an Ih coefficient, except in the Iso-Na-L scenario (see Discussion, below, for an explanation). The β coefficients for each conductance differed considerably by perturbation, suggesting that the choice of perturbation affected the sensitivity of the model to changes in particular conductance parameters. For example, in the RVR scenario, in which pairs of model neurons were chosen randomly in parameter space (Figure 37D), βKd was highest for the cp1 perturbation, and high for chirp, ep2 and ropt. With white noise, however, waveform distance did not depend linearly upon differences in \( g_{Kd} \), and thus there was no \( \beta_{Kd} \) coefficient in the white noise linear fit. This is unsurprising, as the white noise difference landscape was flat along the \( g_{Kd} \) axis (red surfaces, Figure 36 B and D).

The β coefficients also varied considerably by conductance parameter, making it hard to visually compare perturbations to determine which ones produced high correspondence between conductance parameter differences and waveform distance. To facilitate this comparison, the β coefficients for a given conductance parameter were normalized, within each scenario. This was
done by considering all the $\beta$ values for a given conductance parameter in a given scenario as a group, and normalizing them by the maximum within that group. For example, within the RvR scenario, the $\beta_A$ values from the seven linear models made for the seven perturbations were considered together, divided by the largest such $\beta_A$ value, in this case the $\beta_A$ from the white noise perturbation. This was repeated independently for the seven $\beta_{CaS}$ values, the seven $\beta_{KCa}$ values, etc. The results were visualized (Figure 38). Again, there were visible differences between perturbations. The \textbf{chirp}, \textbf{ropt}, \textbf{cp1}, and \textbf{cp2} perturbations were good performers, yielding high $\beta$ values for most currents in most scenarios. White noise was a notably poor performer, with some currents completely absent from the linear models; only the fast currents ($I_{Na}$ and $I_{CaT}$) had high $\beta$ coefficients with white noise.

To summarize this information, each perturbation was assigned a score which reflected the number of conductance parameters successfully fit by the model (i.e., $\beta > 0$), and within this class, the overall F statistic of the model (Table 8). Perturbations with higher scores produced difference landscapes which were more sensitive to change in a greater number of conductance parameters. The \textbf{cp1} composite perturbation had the highest rank, with the \textbf{chirp} and \textbf{rchirp} also ranked highly. Pink noise was the best performer in the random vs. random scenario with leak and sodium conductances present, and performed well in other scenarios. White noise was again a consistently poor performer. Note that the F scores necessarily reflect the linearity of the difference landscapes, as no attempt was made to fit the conductance parameter to $D_W$ relationship using a quadratic or exponential curve which might more appropriately fit the shape of some of the difference landscapes.
Figure 35. Mean waveform distances for perturbations in voltage clamp separation test.

Error bars show mean ± standard deviation. Note different Y axis scale in Iso-Na-L scenario, in which model neurons were chosen randomly from basin of equivalence (E). Variances were high (wide error bars), as waveform distance varied with parameter value differences regardless of perturbation. Differences in means among perturbations were nonetheless often significant, since comparisons were done pairwise.
Figure 36. Example difference landscapes for voltage clamp perturbations.

Two parameter space slices (A, C: CaT vs. KCa; B, D: Kd vs. Na) are shown for two different reference models (A, B: (0,5,4,0,5,125,0,01,200); C, D: (0,1,8,3,50,7,95,0,012,250)); \( g \) for leak, CaT, Cas, A, KCa, Kd, H, and Na, in mS/cm². Reference parameter values are identified in each panel by black vertical lines through minima. Surfaces are colored by perturbation (blue: ropt repeated optimized perturbation; green: rchirp random chirp; red: white noise). The optimized perturbation yielded steeper slopes, as did chirp and cp2 (not shown). White noise produced a steeper slope than rchirp along the \( g_{Na} \) axis, whereas rchirp produced a steeper slope along the \( g_{Kd} \) axis, leading to a crossover of red and green surfaces in B and D. The non-monotonicity of surfaces in A is due to the influence of calcium influx from \( I_{CaT} \) upon \( I_{KCa} \).
Figure 37. β coefficients and residuals for linear models from voltage clamp separation test.

Linear regression models were made to pair_errors data for every perturbation in each scenario (A-E), fitting $D_W$ to absolute differences in conductance parameter values. Mean (circles) and standard error (error bars) are shown for the different β coefficients (left) and intercept β0 (int, right); residual error (res, right) is also shown. When the linear models did not include coefficients for some parameters (e.g., $\beta_{CaT}$ for most perturbations in panel D), no corresponding point was plotted; this indicated that changes to the parameter value did not contribute to differences in $D_W$. Note the different y axis scale in panel E left. */**: Coefficient scaled down 10x (*) or 100x (**) to represent the data on the same axis as the remaining coefficients.
Figure 38. Normalized β coefficients for linear models from voltage clamp separation test.

Results are given for each scenario (A-E). For a given conductance parameter, the β coefficients (Figure 37) for all perturbations were normalized by dividing by the maximum within a group. This improves visibility of β coefficients for conductance parameters in which changes have less impact on $D_w$, such as $\bar{g}_{Na}$ or $\bar{g}_{CaS}$. Negative coefficients are not shown.
Table 7

Rank by Significant Difference in Waveform Distance in Voltage Clamp Perturbation Separation Test

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Perturbation separation rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso-Na-L</td>
<td>ropt (6) &gt; cp2 (5) &gt; chirp (4) &gt; cp1 (3) &gt; rchirp (2) &gt; pink (1) &gt; white (0)</td>
</tr>
<tr>
<td>PvR-Na-L</td>
<td>ropt (6) &gt; cp2, chirp (4) &gt; cp1 (3) &gt; rchirp (2) &gt; pink (1) &gt; white (0)</td>
</tr>
<tr>
<td>PvR-L</td>
<td>chirp, ropt (5) &gt; cp2 (4) &gt; cp1 (3) &gt; white, rchirp (1) &gt; pink (0)</td>
</tr>
<tr>
<td>RvR-Na-L</td>
<td>ropt (6) &gt; cp2, chirp (4) &gt; cp1 (3) &gt; rchirp (2) &gt; pink (1) &gt; white (0)</td>
</tr>
<tr>
<td>RvR</td>
<td>chirp, ropt (5) &gt; cp2 (4) &gt; cp1 (3) &gt; rchirp (2) &gt; white (1) &gt; pink (0)</td>
</tr>
</tbody>
</table>

Perturbations ranked according to significant differences (against all other perturbations) at p<0.05 in post-hoc tests. Number of significant differences is shown in parentheses. For example, in the RvR scenario, ropt and chirp each produced significantly higher $D_w$ than 5 other perturbations, while cp2 was significantly better than 4 other perturbations. When one perturbation ranks higher than another, this does not necessarily mean that the former is significantly better than the latter.
Table 8

Rank by Linear Model Completeness and F-score in Voltage Clamp Perturbation Separation Test

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Perturbation</th>
<th>Parameters Fit</th>
<th>F</th>
<th>n</th>
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<td>CaT A KCa Kd</td>
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<td>ropt</td>
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<tr>
<td>cp1</td>
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<td>leak CaT CaS A Na</td>
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Perturbations ranked by number of conductance parameters successfully fit (i.e., having $\beta > 0$), and by F score, within each scenario. Ranking does not indicate significant difference between model F scores. $n =$ number of point pairs tested (with all perturbations)
Voltage clamp pathfinding performance results

The voltage clamp perturbations were next compared using the **raindrop** program to assess whether pathfinding performance was better with some perturbations than others, in the five different scenarios. Gradient descents were performed repeatedly for randomly chosen target and raindrop points according to scenario, and progress and speed were calculated (**Materials and Methods - Assessing pathfinding performance**). Mean progress (Figure 39) and speed (Figure 40) scores were calculated for each perturbation in each scenario, both overall and per conductance parameter. The mean progress and speed scores generally showed only moderate differences, with relatively large standard deviations. Progress of 90% or more was common, except for white noise. No perturbation yielded good progress with respect to $g_H$. The optimized perturbations performed less well with $g_{Ca}$ and $g_{leak}$. Results for speed were similar to progress, with the optimized perturbations showing somewhat better performance than other perturbations, again excepting $g_{Ca}$ and $g_{leak}$.

An ANOVA with post-hoc test was performed on each scenario to clarify how progress and speed varied by perturbation, either overall or for specific conductance parameters. Significant differences were found in many cases. To clarify these results, a rank was assigned to each perturbation according to the number of significant differences (of greater value) versus other perturbations per scenario, for both progress (Figure 41) and speed (Figure 42). With respect to progress, **chirp**, **rchirp**, and pink noise were consistently highly ranked; **cp2** and **ropt** optimized perturbations had intermediate rank; and **cp1** had low rank. With respect to speed, **cp2** and **ropt** were highly ranked, and **chirp** and **cp1** had low rank. White noise was highly ranked for $g_{Ca}$ and $g_{leak}$ in both progress and speed, but was typically ranked worst in all other cases.
Figure 39. Overall and per-parameter progress in voltage clamp pathfinding test.

Results are given for each scenario (A-E). Progress is the fraction of distance moved towards the target from the raindrop’s initial position. If the raindrop’s final position was farther from the target than its initial position, this was considered a progress of 0. All shows the overall progress in parameter space, using Euclidean distance. Error bars show mean ± standard deviation.
Figure 40. Overall and per-parameter speed in voltage clamp pathfinding test.

Results are given for each scenario (A-E). Speed is the change in parameter value between starting and ending position of the raindrop, divided by the number of steps taken. *All* is Euclidean distance between raindrop start and end positions, divided by steps. Only movement towards the target was considered; a raindrop which moved away from the target was assigned speed 0. Error bars show mean ± standard deviation.
Figure 41. Perturbation progress ranks in voltage clamp pathfinding test.

A perturbation’s rank, within a given scenario (A-E) and for a given conductance parameter, reflects the number of significant differences of greater mean progress versus other perturbations in the post-hoc test. Progress was also assessed for Euclidean distance in parameter space (All).

The sum of all parameter ranks was calculated (TOT); the total ranks were generally in agreement with the Euclidean distance ranks. Note that the differences in means, though significant, were often small (Figure 39); see Discussion, below. Where no such significant differences were found (e.g., H conductance parameters in most scenarios), the corresponding bars are not plotted.
Figure 42. Perturbation speed ranks in voltage clamp pathfinding test.

A perturbation’s rank, within a given scenario (A-E) and for a given conductance parameter, reflects the number of significant differences of greater mean speed versus other perturbations in the post-hoc test. Speed was also assessed for Euclidean distance in parameter space (All). The sum of all parameter ranks was calculated (TOT); the total ranks were generally in agreement with the Euclidean distance ranks.
Current clamp separation performance results

Eight 174 s current clamp perturbations were compared using the pair_errors program in the five different scenarios. Again, all data were gathered and analyzed separately for each scenario. Mean waveform distances were calculated for each perturbation (Figure 43). In the RvR scenario, in which the presence of a leak current limited amplitude and prevented rhythmic activity, mean waveform distances were small. In all other scenarios, results were mixed. An analysis of variance (ANOVA) with Tukey’s HSD post-hoc test was performed for each scenario, establishing that $D_w$ differed significantly with different perturbations. Ranking the perturbations by the number of significant differences above all other perturbations yielded inconsistent results (Table 9). The phase-locking pulse gave poor separation performance in the Iso-Na-L scenario as expected, and in the RvR scenario, but good performance in all other scenarios, in which at least one of the pair of neurons was rhythmic. Unlike the voltage clamp results, the noisy perturbations – specifically nchirp and white noise – performed well, as did chirp, whereas rchirp and pink noise performed poorly. Two dimensional parameter space slice visualizations (Figure 44) showed pathological difference landscapes for most perturbations, with non-monotonicity and local minima.

As with voltage clamp, linear regression models were constructed for each perturbation to correlate waveform distance with the differences in conductance parameter values (Figure 45). Residuals were generally high compared to $\beta$ coefficients, and non-monotonicity was common. Again, the presence of leak conductance in RvR scenario reduced model membrane voltage amplitude and thus sensitivity of waveform distance to changes in most conductance parameters. Normalized $\beta$ values were calculated (Figure 46); no perturbation was consistently better across conductance parameters and scenarios. Ranking by number of fitted conductance parameters and
F scores (Table 10) did not show consistent results, though, white and pink noise were often highly ranked.

Figure 43. Mean waveform distances for perturbations in voltage clamp separation test. Results are shown for all scenarios (A-E). Error bars show mean ± standard deviation. Despite the large variances, differences among some means are significant, as comparisons are performed pairwise. cp1 and ropt refer to the cp1-cc and ropt-cc current clamp perturbations.
Figure 44. Example difference landscapes for current clamp perturbations.

Two parameter space slices are shown (A-C: Ca_T vs K_Ca; D-F: K_d vs Na) are shown for a single reference model with coordinates (0,5,4,0,5,125,0.01,200); $\bar{g}$ values for leak, Ca_T, Ca_S, A, K_Ca, K_d, H, Na, in mS/cm$^2$. Difference landscape surfaces are shown for pink noise (A,D), chirp (B,E), and ropt-cc optimized perturbation (C,F). Surfaces are colored by waveform distance. Gaussian smoothing was applied to improve visibility. Unlike voltage clamp, where membrane voltage is constrained by the clamping process, a feedback loop exists between $V_m$ and conductance activation state. Accordingly, the current clamp difference landscapes are often non-monotonic, with local minima (wrinkles and ridges).
Figure 45. β coefficients and residuals for linear models from current clamp separation test.

Linear regression models were made to pair_errors data for every perturbation in each scenario (A-E), fitting $D_w$ to absolute differences in conductance parameter values. Mean (circles) and standard error (error bars) are shown for the different β coefficients (left) and intercept $\beta_0$ (int, right); residual error (res, right) is also shown. When the linear models did not include coefficients for some parameters, no corresponding point was plotted; this indicated that changes to the parameter value did not contribute to differences in $D_w$. Note different y axis scale in panel E. */***: Coefficient scaled down 10x (*) or 1000x (**) to represent on the same axis as the remaining coefficients. cp1 and ropt refer to the cp1-cc and ropt-cc current clamp perturbations.
Figure 46. Normalized β coefficients for linear models from current clamp separation test. Results are given for each scenario (A-E). For a given conductance parameter, the β coefficients for all perturbations were normalized by dividing by the maximum within a group. This improves visibility of β coefficients for conductance parameters in which changes have less impact on $D_w$, such as $\bar{g}_{Na}$ or $\bar{g}_{Ca5}$. Negative coefficients are not shown. cp1 and ropt refer to the cp1-cc and ropt-cc current clamp perturbations.
Table 9

Rank by Significant Difference in Waveform Distance in Current Clamp Perturbation Separation Test

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Perturbation separation rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso-Na-L</td>
<td>white (7) &gt; chirp (6) &gt; nchirp (5) &gt; pulse (4) &gt; ropt (3) &gt; cp1 (2) &gt; rchirp (1) &gt; pink (0)</td>
</tr>
<tr>
<td>PvR-Na-L</td>
<td>pulse (7) &gt; white, ropt (5) &gt; cp1 (4) &gt; nchirp (3) &gt; chirp (2) &gt; rchirp (1) &gt; pink (0)</td>
</tr>
<tr>
<td>PvR-L</td>
<td>nchirp (7) &gt; pink (6) &gt; rchirp (5) &gt; cp1 (4) &gt; pulse, chirp (2) &gt; ropt (1) &gt; white (0)</td>
</tr>
<tr>
<td>RvR-Na-L</td>
<td>pulse (7) &gt; ropt (6) &gt; white (5) &gt; cp1 (4) &gt; nchirp (3) &gt; chirp (2) &gt; rchirp (1) &gt; pink (0)</td>
</tr>
<tr>
<td>RvR</td>
<td>nchirp (6) &gt; chirp (4) &gt; pink, cp1 (2) &gt; rchirp (1) &gt; white, pulse, ropt (0)</td>
</tr>
</tbody>
</table>

Perturbations ranked according to significant differences at p<0.05 in post-hoc tests. **cp1** and **ropt** refer to the **cp1-cc** and **ropt-cc** current clamp perturbations.
Table 10

Rank by Linear Model Completeness and F-score in Current Clamp Perturbation Separation Test

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Perturbation</th>
<th>Parameters Fit</th>
<th>F</th>
<th>n</th>
</tr>
</thead>
<tbody>
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<tr>
<td>RvR</td>
<td>pulse</td>
<td>CaS KCa Na leak</td>
<td>93.03</td>
<td>16000</td>
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<td>chirp</td>
<td>CaS leak</td>
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<td></td>
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<td>nchirp</td>
<td>CaS leak</td>
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<td>pink</td>
<td>CaS leak</td>
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<td></td>
<td></td>
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<td>CaS leak</td>
<td>122</td>
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<td></td>
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</tbody>
</table>
Table 10: Continued

Perturbations ranked by number of parameters successfully fit (i.e., having $\beta > 0$), and F score, per scenario. Ranking does not indicate significant difference between model F scores. $n =$ number of point pairs tested. cp1 and ropt refer to the cp1-cc and ropt-cc perturbations.

Current clamp pathfinding performance results

The current clamp perturbations were compared using the raindrop program to assess pathfinding performance in the five different scenarios, as was done with voltage clamp. Mean progress (Figure 47) and speed (Figure 48) scores were calculated for each perturbation in each scenario, both overall and per conductance parameter. Results were inconsistent and variances were high, though pink noise and rchirp generally showed better scores. Progress scores were typically low, 20% or less, due to the frequent local minima in the difference landscapes. In contrast to voltage clamp results, progress along the $\overline{g_{ii}}$ axis was typically better than progress for other conductance parameters (see Discussion below). The optimized perturbations did not generally result in high progress or speed scores; white and pink noise yielded better results than ropt.

An ANOVA with post-hoc test was performed on each scenario to evaluate how progress and speed varied by perturbation, either overall or for specific conductance parameters. Far fewer significant differences were found than with voltage clamp. Ranks were assigned to each perturbation as with voltage clamp, for both progress (Figure 49) and speed (Figure 50). Pink noise and the rchirp random chirp were the most highly ranked in progress, and were also highly ranked in speed. The chirp also had high speed ranks, but its progress rank was considerably lower than either pink noise or rchirp.
Figure 47. Overall and per-parameter progress in current clamp pathfinding test.

Results are given for each scenario (A–E). Progress is the fraction of distance moved towards the target from the raindrop’s initial position. If the raindrop’s final position was farther from the target than its initial position, this was considered a progress of 0. All shows the overall progress in parameter space, using Euclidean distance. Error bars show mean ± standard deviation. cp1 and ropt refer to the cp1-cc and ropt-cc current clamp perturbations.
Figure 48. Overall and per-parameter speed in current clamp pathfinding test.

Results are given for each scenario (A-E). Speed is the change in parameter value between starting and ending position of the raindrop, divided by the number of steps taken. *All is Euclidean distance between raindrop start and end positions, divided by steps. Only movement towards the target was considered; a raindrop which moved away from the target was assigned speed 0. Error bars show mean ± standard deviation. *cp1 and *ropt refer to the *cp1-cc and *ropt-cc current clamp perturbations.
Figure 49. Perturbation progress ranks in current clamp pathfinding test.

A perturbation’s rank, within a given scenario (A-E) and for a given conductance parameter, reflects the number of significant differences of greater mean progress versus other perturbations in the post-hoc test. Progress was also assessed for Euclidean distance in parameter space (*All*). The sum of all parameter ranks was calculated (TOT). *cp1* and *ropt* refer to the *cp1-cc* and *ropt-cc* current clamp perturbations.
Figure 50. Perturbation speed ranks in current clamp pathfinding test.

A perturbation’s rank, within a given scenario (A-E) and for a given conductance parameter, reflects the number of significant differences of greater mean speed versus other perturbations in the post-hoc test. Speed was also assessed for Euclidean distance in parameter space (*All*). The sum of all parameter ranks was calculated (TOT). *cp1* and *ropt* refer to the *cp1-cc* and *ropt-cc* current clamp perturbations.
Discussion

*Optimized perturbations*

The performance of the evolution strategy was improved considerably by nonstandard extensions – genetic drift, and changes to tuning coefficients – and by careful adjustment of the fitness function. Once tuned, the evolution strategy rapidly transformed random waveforms into voltage clamp perturbations which could elicit specific currents against a background of others in the Prinz model (Prinz et al., 2003). The fitness function used was designed to maximize the amplitude and duration of the target current, without penalizing for other currents when the target current was not active, with the expectation that this would lead to appropriately rhythmic perturbations where the target current inactivated or where competing currents could mask its appearance. Such rhythmic character rapidly evolved in the nontrivial cases (*Figure 31* and *Figure 32*) with timing appropriate to the channel kinetics. A common motif that appeared in many perturbations was a section of hyperpolarizing potential which removed inactivation, followed by a depolarizing pulse, sometimes ending with a steep hyperpolarizing ramp, presumably to increase the driving force on the ion in question while the channel remained open. The lack of evolutionary pressure in the hyperpolarizing regions was apparent; these regions typically retained their random ramp character. It is likely that the amplitudes of the perturbation sections are dependent upon the voltage dependence of the conductance equation parameters, i.e., the $v_{1/2}$ and slope parameters of the activation ($m$) and inactivation ($h$) gates. Similarly, the timing of the waveform regions likely depends upon the kinetic parameters of the conductance equations, i.e., those governing the $\tau_m$ and $\tau_h$.

A different fitness function choice could have designed a single perturbation which would be good for eliciting all of the currents, e.g., by assigning a fitness score based upon the volume of phase space explored. We chose instead to maximize one current at a time, as this was
easier to implement and evaluate. Furthermore, an eventual goal of the stimulation was stimulating live neurons in a way which could elicit responses based upon conductance complement, and temporally separating the different conductance-optimized sections would simplify later analysis (Chapter 4).

Voltage clamp results

The optimized perturbations and several additional perturbations were tested for separation and pathfinding performance in voltage clamp. For most voltage-dependent conductances, the state of the activation and inactivation gates depends solely upon their previous state and upon membrane potential. As the initial conditions of the activation and inactivation gates are defined during integration, the response of each voltage-dependent current depends only upon the voltage clamp signal, and is independent of the amplitude of other currents. The notable exception in the Prinz model is $I_{K_{Ca}}$, which is sensitive both to $V_m$ and to intracellular calcium concentration, itself dependent upon the activity of calcium conductances. Except for $I_{K_{Ca}}$, then, in a compartment with perfect voltage clamping, for a given perturbation, as a current’s $\bar{g}$ parameter value is changed, the amplitude of that current (and only that current) should vary linearly with the changing $\bar{g}$, as the activation and inactivation gates will not change. As these tests were conducted in a single-compartment model which assumed perfect voltage clamping, we expected that a perturbation’s performance in both pathfinding and separation would reflect its ability to maximally evoke the voltage-dependent currents.

As hypothesized, the optimized perturbations performed extremely well in separating model neurons on the basis of conductance complement, and reasonably well at doing so with a linear relationship between changing conductance parameters and changing output. Visualizations of two-dimensional slices of the difference landscape for various perturbations in voltage clamp around a bursting reference neuron model demonstrated the higher waveform
distance values yielded by the use of the optimized perturbation (*Figure 36*). The repeated optimized perturbation, $\text{ropt}$, produced the best separation overall. The chirp was also quite good, presumably because different frequency sections corresponded with the kinetics of different channels. White noise was particularly bad at activating most currents, notably excepting the fast calcium and sodium currents, and thus the white noise perturbation did not separate model neurons as effectively as other perturbations.

The relationship between response distance and conductance parameter distance was most linear in the combined $\text{cp1}$ protocol which incorporated sections of chirp and pink noise. This was initially somewhat surprising. The higher rank of $\text{cp1}$ is in part caused by lower residuals in its linear model compared to $\text{cp2}$ or $\text{ropt}$, possibly due to the increased contribution of $I_{\text{KCa}}$ with $\text{cp2}$ and $\text{ropt}$. The parameter space slice landscapes including $I_{\text{KCa}}$ and either $I_{\text{CaS}}$ or $I_{\text{CaT}}$ were notably non-monotonic with $\text{ropt}$ in particular (*Figure 36A*), reflecting the dependence of the calcium-dependent potassium current upon intracellular calcium influx due to the calcium current. Also, in the *Iso-Na-L* scenario, $\text{cp1}$ yielded a linear model which included a $\beta_H$ coefficient (*Figure 38E*); no other perturbation yielded a linear model included $\beta_H$, nor did any other scenario (see below).

With respect to pathfinding, most perturbations except for white noise were good for most scenarios. Progress of 90% or more towards the target conductance parameters was typical (*Figure 39*). The chirp and rchirp and pink noise were highest ranked in progress, whereas the optimized perturbations were highest ranked in speed, likely due to the steeper slopes of the difference landscapes. The differences in progress between perturbations, though slight, were typically statistical significant. The higher progress scores for the non-optimized perturbations were initially surprising, as the difference landscape should be smooth and generally monotonic. However, careful examination of the individual progress values for each conductance (*Figure 39*)
revealed lower progress for the optimized perturbations in Ca_T, Ca_S, and K_Ca. It is possible that eliciting greater amounts of these currents may be a liability in the Nelder-Mead simplex gradient descent test, by producing greater non-monotonicity (e.g., as in Figure 36A).

Overall these results demonstrate that it is possible to use an evolution strategy to empirically optimize a perturbation for its ability to elicit large amplitudes of voltage-dependent conductances in voltage clamp. These optimized perturbations provide excellent performance in causing model neurons with different parameters to produce different responses, and good performance in pathfinding using local gradient descent. This suggests that these perturbations may be useful in identifying and characterizing real neurons based upon response to voltage clamp. The large amplitude of voltage-dependent currents is particularly important, as some of these currents may be small and difficult to measure against background noise in real neurons.

**Current clamp results**

In voltage clamp, the stimulus waveform controls the gating particle states. In current clamp however there is feedback between the membrane potential and the state of the activation and inactivation gates, and thus the neuron is free to move about in phase space in a way that depends upon its maximal conductance parameter values. As such, the evolved perturbations are highly specific to the conductance parameters of the model neuron for which they were designed. A perturbation which might, for example, elicit a large amount of I_A current by driving the neuron into resonant rhythmic activity, might not do so if g_H were substantially changed, altering the resonant frequency of the neuron enough to prevent resonance. Furthermore, in the presence of a leak conductance, the spontaneous activity of the neuron would be reduced or eliminated. Thus, it was not expected that the current clamp perturbations would yield good performance.

This was confirmed in both separation and pathfinding tests. The optimized perturbations did not produce good separation in any scenario, and the noisy perturbations (white
noise, pink noise, randomized chirp, and noisy chirp) gave better results. The phase-locking pulse (i.e., free-running conditions) also gave good separation results with rhythmic model neurons not in a basin of equivalence. Pink noise produced the most linear difference landscape in most scenarios. Pathfinding was also poor when using the optimized perturbations, whereas pink noise and the randomized chirp gave considerably better results, being more highly ranked in progress in most scenarios. Overall these results suggest that noise based perturbations offer superior performance in current clamp experiments, and that empirically optimizing perturbations to work in current clamp may in general be difficult.

*Impact of $I_H$ maximal conductance on waveform distance*

In voltage clamp, changes to $\overline{g}_H$ did not have appreciable effect upon waveform distance. In the voltage clamp separation tests, only the linear models made in the Iso-Na-L scenario included a $\beta_H$ term, which was very small (*Figure 37*), indicating that waveform distance was not sensitive to $\overline{g}_H$ changes. In voltage clamp pathfinding tests, progress and speed towards the target in $\overline{g}_H$ were both substantially lower compared to other conductance parameters. In current clamp, however, the opposite effects were observed. In linear models constructed in the current clamp separation tests, $\beta_H$ was often large, regardless of perturbation or scenario. In current clamp pathfinding tests, progress and speed towards the target in $\overline{g}_H$ were also often large.

The reason for these somewhat paradoxical results is the nature of the $I_H$ current in the Prinz model, and the magnitude of this current in the pacemaker neurons used in this research. Prinz et al conducted a course search of parameter space to classify neurons by type, and used a maximum value of $\overline{g}_H$ of 0.05 mS/cm$^2$ in this search (Prinz et al., 2003). In particular, they identified parameter values of bursting pacemaker neurons with similar activity (see Figure 10 of Prinz et al., 2003), all with no leak conductance and with $\overline{g}_H = 0.01$ mS/cm$^2$. These model
neurons were used for the \textbf{PvR} scenarios (Table 6). The model neurons used in the \textbf{Iso-Na-L} scenario were taken from a basin of equivalence of one of these pacemakers, using the vector and floodfill method (Chapter 2) to explore a reduced parameter space excluding leak. Only in the \textbf{RvR} scenario did any model neuron selected for comparison contain a leak conductance.

In the absence of the leak conductance, the sole contribution to the input resistance of the model neurons at hyperpolarized potentials is $I_{\text{H}}$. As such, very small changes to $g_{\text{H}}$ can have a large effect upon hyperpolarized membrane potentials in current clamp. Furthermore, because $I_{\text{H}}$ alone is active during the hyperpolarized phase of the rhythm, small changes in $g_{\text{H}}$ also have disproportionately large effects on the frequency of the rhythm, and thus, upon waveform distance calculations (Figure 51). However, the actual magnitude of this current is still very small in comparison to the other voltage-dependent currents, whose maximal conductances are 2-4 orders of magnitude larger. Thus, in voltage clamp, small changes to $g_{\text{H}}$ are unlikely to have a noticeable effect upon clamp current, and thus, waveform distance.

More generally, the equations governing the kinetics and voltage-dependence of the $I_{\text{H}}$ conductance in the Prinz model are not necessarily appropriate for pyloric neurons (Figure 58 in Chapter 4). If this is the case, then it is possible that attempting to replicate the behavior of real neurons by parameter search could yield parameter values which are not correct. One effect of increasing $g_{\text{H}}$ in a bursting pacemaker is to increase burst (slow-wave) frequency. If the real pyloric $I_{\text{H}}$ has considerably slower kinetics than the Prinz model $I_{\text{H}}$, then the correct $g_{\text{H}}$ to produce a specific frequency could be greater than the model predicts (Figure 52). This would in turn permit a greater leak current to be present while maintaining rhythmicity.
Figure 51. Small changes to $I_{\text{H}}$ conductance have large effect on spontaneous activity.

Burst frequency increases in a pacemaker neuron (parameter values from first row of Table 6) as $g_{\text{H}}$ increased from 0.01 (A) through 0.1 (C), 1 (E), 10 (G), and 100 (I) mS/cm$^2$. As these model neurons have no leak, $I_{\text{H}}$ provides the only source of conductance at hyperpolarized potentials. In the presence of even a small leak current, 0.01 mS/cm$^2$, rhythmic activity is abolished, only being restored at higher $g_{\text{H}}$ values (B,D,F,H,J: $g_{\text{H}}$ same as in corresponding left panels). A parameter search in which $g_{\text{H}}$ is bounded at a low value may thus be less likely to identify rhythmically active model neurons with appreciable leak current. Model neurons were perturbed with an 0.5 s current pulse of 1 nA starting at 500 ms.
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Disclosures

The authors have no conflicts of interest.
CHAPTER 4: EMPIRICALLY OPTIMIZED PERTURBATIONS CLASSIFY NEURONS

WITHOUT A PRIORI FEATURE IDENTIFICATION

Author Contributions

The research in this chapter was performed to evaluate the performance of the perturbations identified in the previous chapter in real neurons. W. E. W. designed the experiments, wrote the software, and performed all analysis. J. B. T. performed all lobster dissections. J. B. T. and W. E. W. identified pyloric neurons. W. E. W. conducted the voltage and current clamp experiments. S. L. H contributed to interpretation of results. W. E. W. prepared all figures and wrote the manuscript. S. L. H. and all dissertation committee members assisted in editing the manuscript and figures.

Abstract

Prior research has identified that empirically optimized perturbations, designed using a genetic algorithm to elicit distinctive responses of voltage-dependent currents, can produce superior performance in characterizing model neurons in silico. A composite perturbation including these optimized perturbations was evaluated in real pyloric neurons. This perturbation caused pyloric neurons to produce distinctive responses. Hierarchical clustering performed using waveform distance on their responses classified these neurons into categories which recapitulate their anatomical types. This clustering was statistically significant, and was robust to change in linkage method.
Introduction

In previous chapters it was established that model neurons can produce quantitatively similar free-running behavior despite having different conductance parameter values, and that these parameter values range widely throughout parameter space (Chapter 2). It was then shown that when appropriately perturbed, similarly behaving neurons with different conductance parameter values will separate, generating different responses (Chapter 3). A series of voltage clamp perturbations were empirically optimized using an evolution strategy for maximizing the amount of each voltage dependent current against the background of other currents. When compared to other perturbations such as chirp or white noise, these perturbations showed good performance for characterizing model neurons on the basis of conductance complement, as well as for producing a difference landscape which facilitates determining the conductance parameters of an unknown neuron by heuristic methods.

The performance of these perturbations has not however been established in real neurons. The Prinz pyloric neuron model (Prinz et al., 2003), which was utilized to develop and establish the optimized perturbations, was an abstraction of real decapod crustacean pyloric neurons. As such, it simplified many of the real neurons’ characteristics. It was implemented in a single isopotential compartment, whereas many real neurons have highly complex morphology (Thuma et al., 2009) and are not generally isopotential (Chapter 6). It assumes a set of conductances whose only free parameters are the $\bar{g}$ (maximal conductance) values, whereas it is known that in real neurons, the ion channels underlying these conductances can vary in kinetics and voltage dependence (Ouyang et al., 2007). It posits a particular set of conductances, which may or may not be complete; notably, real pyloric neurons may include a persistent sodium conductance which is not included in this model. Finally, it assumes perfect voltage clamp and, because of isopotentiality, no space clamp limitations, whereas in real neurons, voltage clamping can be
imperfect, especially in regions distal to the soma. In these imperfectly clamped regions, the response of the neuron depends not merely upon the driving signal but also upon its intrinsic activity, in much the same way as current clamp. This is a concern because the empirically optimized perturbations did not demonstrate high performance in current clamp conditions (Chapter 3).

The performance tests in the neuron model relied upon knowing the underlying conductance parameters of the model neurons. In real neurons, these parameters are unknown, so alternative tests are needed. The analogue of pathfinding is determining conductance parameters which will cause a model neuron to produce identical output to a real neuron regardless of perturbation. This could be done by stimulating a real neuron with an appropriate perturbation, then adjusting the conductance parameters of the model until its activity matches the real neuron, using a heuristic such as an evolution strategy (Hobbs and Hooper, 2008). This is a major goal in computational neuroscience, and achieving it has thus far proven difficult, suggesting that existing models may be incomplete, the perturbations used may be insufficient, or that the number of free parameters may be too large for the electrical activity of the neuron alone to constrain.

Separation could however be considerably easier to evaluate. A perturbation with good separation performance will cause neurons with similar conductance parameters to respond similarly, and those with different conductance parameters to respond differently. In *Panulirus interruptus*, and many other invertebrates, neurons in some networks are classified into types according to which muscles they innervate, and identified based upon their unperturbed intracellular or extracellular activity. This activity, and thus the identification, may reflect the intrinsic properties of the neurons, their network connectivity, or both. If the intrinsic properties determine or strongly contribute to the activity of these neurons, then a perturbation with good
separation performance may cause these neurons to respond in a way that recapitulates their type. A separation performance test in real neurons would thus be to eliminate driving input from other neurons, perturb them, classify them according to their responses, and determine whether this classification recapitulates their identification into type by traditional means.

There are several possible concerns which could prevent perturbed activity from recapitulating types. First, if these neurons’ unperturbed activity is more dependent upon their driving input or network properties than their intrinsic properties, then their responses to perturbation may be largely unrelated to their natural activity. In this case classification by perturbed activity may correctly reflect underlying conductance parameter values, but verifying this would require experiments to determine these properties, such as chemical blockade to isolate and measure specific ionic conductances. Such chemical blockade is often irreversible, so it is not possible to characterize multiple conductance parameters in a single neuron.

Assuming the neuron’s activity is due to intrinsic properties, a second problem is that these properties could vary widely within neurons of a given type. It is known that both model and real neurons can produce similar activity despite having different conductance parameters (Chapter 2). Real neurons presumably maintain their activity by feedback mechanisms which dynamically adjust their intrinsic properties, but it is not known whether this occurs in a way which constrains these properties to a relatively small region of parameter space. If not, then even if the perturbation does produce responses which reflect the underlying conductance parameters, neurons of a given type may produce widely different responses when perturbed, again preventing classification which recapitulates types.

A third concern is that the intrinsic properties contributing to the neuron’s activity may include properties other than the voltage-dependent conductances present in the model used to develop and assess the perturbations. The optimized perturbations were developed in a model
which included a persistent sodium conductance, as it was suspected that this conductance may be present in pyloric neurons despite its absence in the Prinz model. However, there may be other conductances in pyloric neurons which affect their activity. Other intrinsic properties which may affect spontaneous or driven activity also include morphological variations, differences in input resistance, and somatic shunting during voltage clamping.

A final problem is that the properties of the neuron may not be static. Assuming the properties of the neuron are dynamically maintained based upon some measure of activity, such as intracellular calcium concentration (Liu et al., 1998), then these properties should begin to vary as soon as afferent input to the neuron is removed. The experimental process itself may also result in changes to neuron properties, if for example the sharp microelectrodes puncture the endoplasmic reticulum resulting in calcium release, or if the cell membrane does not adequately seal after impalement resulting in change in intracellular ionic concentrations. These concerns at least can be addressed, by limiting the time between impalement and perturbation, and the time between removal of afferent input and perturbation.

Overview of research

To assess separation performance, we identified pyloric neurons in the stomatogastric ganglion of *Panulirus interruptus* based upon extracellular activity, isolated them with chemical blockade, subjected them to voltage clamp using the composite optimized perturbation cp1 (Chapter 3), classified them by their responses, and then compared this classification to their previously identified types. We anticipated that the classification could fail to recapitulate type should any of the previously mentioned concerns apply. However, if their perturbation-based classification were to recapitulate neuron type, this would be evidence that the optimized perturbation is effective in separating real neurons based upon intrinsic properties. The optimized perturbation contains temporally separated segments each optimized for a particular conductance.
If the conductance complement of different neuron types differs, then these different segments should stimulate different neurons in different ways, e.g., with neurons of one type showing heightened responses to some segments, and neurons of another type to other segments. This should facilitate classification by simple waveform distance comparisons in each segment.

To minimize concerns about intrinsic properties other than voltage dependent conductances, we used either of two different techniques. First, we normalized the current response waveforms of the neurons by current amplitude and offset, thus reducing the influence of differences in input resistance and resting potential; these were referred to as the **transformed response waveforms**. Second, we removed the passive component of each response waveform by fitting a passive model to the waveform over a region of mostly passive activity, and then subtracting the passive model’s response to the perturbation; these were referred to as the **residual active waveforms**. To address concerns about possible drift of intrinsic properties over time, we minimized the amount of time between impalement and perturbation, and repeated the perturbations after a time delay to assess whether such drift had occurred.

Response waveforms were then compared against each other in several steps to assess whether the responses would recapitulate types. First, the waveform distances between each set of recordings were assessed with an ANOVA and post-hoc test to determine whether the waveform distance between neurons within a type was lower than the waveform distance across types. Then, the waveform distances were clustered using a hierarchical clustering algorithm, which repeatedly chooses the closest two entities (by waveform distance), merges them into a single unit, and then repeats, until everything is clustered together. Next, the statistical validity of the clustering was assessed using bootstrapping. Finally, the sections of the response waveform during which the optimized perturbation was active were isolated, and divided by segment, each corresponding to a voltage-dependent conductance. The mean voltage clamp current in each
segment in each waveform was calculated, and statistical models were made to determine whether the various neuron types responded differently in different segments.
Materials and Methods

Electrophysiology

Panulirus interruptus were obtained from Marinus Scientific (Newport Beach, CA) and kept at 10-13°C in aquaria with circulating artificial seawater. Dissection was standard (Selverston et al., 1976). Preparations were perfused with P. interruptus saline (479 NaCl, 12.9 KCl, 13.7 CaCl₂, 3.9 Na₂SO₄, 10 MgSO₄, 10.9 dextrose, 11.1 Tris, 5.1 maleic acid, all in mM; pH adjusted to 7.5-7.6; Sigma or Fisher Scientific) and held at 10-13°C throughout the experiment. Extracellular recordings were made using an A-M Systems differential amplifier and bipolar stainless steel electrodes insulated with petroleum jelly. Intracellular recordings were made with an Axoclamp 2B and glass sharp microelectrodes pulled on P-87 Flaming/Brown micropipette puller (Sutter Instruments) and filled with 2.5 M KAc, 20mM KCl (resistance 10-20M). Lateral Pyloric (LP), Pyloric (PY), Pyloric Dilator (PD), Ventricular Dilator (VD), Inferior Cardiac (IC), and Anterior Burster (AB) neurons were identified by comparing intracellular activity to pyloric motor neuron activity in the lateral and medial ventricular nerves.

After neuron identifications, a sucrose block was applied to the stomatogastric nerve, which carries input from the rest of the nervous system to the stomatogastric ganglion. Preparations were bathed in P. interruptus saline containing 0.1 uM tetrodotoxin (TTX) and 10uM picrotoxin (PTX) to isolate the neurons by blocking fast sodium spiking and synaptic current. This abolishes any spontaneous activity in these neurons, leaving them stable at resting potentials near -55 mV. Voltage and current clamp were performed with two sharp glass microelectrodes, one used to measure voltage and the other to pass current. The Axoclamp 2B amplifier was used in both voltage clamp and bridge mode. Data were acquired with a Cambridge Electronic Design Power1401 intracellular amplifier and the Spike2 software, and sampled at 10 kHz. Because neurons had different input resistances, the current clamp command
signal was amplified or attenuated using a Brownlee Precision Model 440 instrumentation amplifier. This allowed the current clamp waveform amplitude to be adjusted to drive the neuron between -100 mV and -5 mV regardless of its input resistance. After impalement, neurons were allowed to settle for at least 5 min, and until membrane potential was stable for 2 min. Square wave pulses of 0.5 Hz ranging from -0.2 nA to 1 nA were applied, and gain on the signal amplifier increased or decreased until the current command caused the membrane potential to range maximally within -5 mV and -100 mV, without exceeding it. The same current clamp gain was then used for every current clamp perturbation in a given neuron, regardless of changes in the neuron's input resistance. Actual clamp current after signal amplification was recorded by Spike2.

The Axoclamp was then placed into voltage clamp mode at the resting potential of the neuron and Axoclamp voltage clamp gain was raised to 25. Depolarizing steps were made in 10 mV increments up to -5 mV to assess voltage clamp stability; in the event stability could not be achieved the neuron was discarded. The neuron was then clamped at -50 mV for 20 s, and the voltage clamp perturbation was applied. The Axoclamp was then placed into bridge mode and the neuron was allowed to rest for 1 m. The current clamp perturbation was then applied at the previously determined signal amplifier gain. This was followed by 5 m of rest, and a second set of voltage and current clamp perturbations applied as before. In some neurons, third and subsequent sets of voltage and current clamp perturbations were applied, again with 5 m of rest between sets. The current clamp data were not used except to note slow voltage-dependent phenomena which did not fit the dynamics of the Prinz model currents.

The waveform recordings were then processed by a custom Spike2 script and saved as binary data files comprising time, voltage, and current values, sampled at 10 kHz. Metadata describing experiment date, neuron type and number in the ganglion, stimulation type and set
number, time since impalement, and time since application of TTX and PTX were written to a
database maintained with the binary recordings. Recordings in which obvious artifacts occurred,
in which electrode drift in excess of 5 mV occurred during the experiment, or in which the
neuron's input resistance changed appreciably during a single perturbation, were discarded.

Empirically optimized perturbations

The composite optimized voltage clamp perturbation \textbf{cp1} was used to stimulate neurons
in voltage clamp. This perturbation contained four \textit{sections}, including two identical repeats of the
conductance optimized perturbation, a chirp section, and a pink noise section (\textit{Figure 29}). The
conductance optimized perturbation section itself contained eight individual \textit{segments}, each
corresponding to a different voltage-dependent current (\textit{Figure 33}) in the Prinz+\textit{I\textsubscript{NaP}} model
(\textit{Chapter 3 - Materials and Methods - Pyloric neuron simulations and distance metrics}). Each
such segment was empirically designed using an evolution strategy which mutated a random
waveform to maximize the amount of the desired voltage-dependent current against the
background of other currents. Holding potentials were inserted between segments, and between
sections, to allow the neuron to achieve a steady-state condition before the next segment began.
The \textbf{cp1} perturbation was 200 s total duration, ranging from -100 mV to -5 mV, with dV/dt
limited to 10 mV/ms to avoid potentially damaging clamp currents or the induction of ringing in
the voltage clamp circuitry.

The \textbf{cp1} composite perturbation was chosen over the \textbf{cp2} perturbation because the \textbf{cp1}
perturbation included longer holding potentials between segments, and it was suspected that real
neurons may require longer settling times to achieve steady state conditions. Furthermore, the
\textbf{cp1} perturbation had demonstrated superior performance to either \textbf{cp2} or \textbf{ropt} in the separation
regression tests, with more parameters included in the models, and more waveform distance
accounted for in the \textbf{RvR} scenario (\textit{Chapter 3 – Results – Voltage Clamp Separation Results}).
This scenario was considered appropriate for pyloric neurons, which do not show spontaneous activity when isolated, and which contain a leak conductance. Additionally, if regions of the dendritic arbor were electrotonically distant from the soma, they would not be completely clamped, leading to some feedback between ionic currents and local membrane potential in those regions, much as occurs during current clamp. Pink noise and chirp performed better than the optimized perturbation in current clamp separation tests (Chapter 3 - Results - Current clamp separation performance results), and the \text{cp1} composite perturbation contained pink noise and chirp sections.

\textit{Waveform distance calculation}

When response waveform traces from two neurons were compared, waveform distance, \(D_w\) was used:

\[
D_w(r_1, r_2) = \frac{1}{d} \left( \int_0^d |r_1(t) - r_2(t)|^p \right)^{1/p}
\]

where \(r_1\) and \(r_2\) are the two response waveforms being compared, \(d\) is the total duration.

Euclidean distance \((p = 2)\) was used exclusively throughout this research. This magnified larger differences between the waveforms, thus reducing the influence of the inevitable noise present in recordings from live neurons. The first and last 100 ms of waveforms were not used in waveform distance calculations, to allow for comparison of waveforms which were slightly different in length or which were time shifted to compensate for timing errors.

\textit{Response waveform alignment}

The timing of the command waveform and recording process was imperfect due to the limitations of the data acquisition equipment. Timing errors occasionally appeared in the form of slight delays between the time of the start marker in the recording, and the time at which the current waveform trace showed a response to the voltage clamp signal. To compensate for these
timing errors, the response waveforms were time-aligned before comparison. One of the response waveforms was arbitrarily chosen as a reference waveform, $r_{ref}$, and all other waveforms were aligned against it. A waveform $r_x$ was aligned against $r_{ref}$ by time shifting $r_x$ by $\tau$ ranging from -10 ms to 10 ms in 0.1 ms steps, calculating waveform distance over the first 40 seconds of the recording:

$$D_w(r_x, r_{ref}) = \frac{1}{40 \int_0^{40} |r_{ref}(t) - r_x(t + \tau)|^2}$$

The $\tau$ at which waveform distance was minimized was selected, and the response waveform $r_x$ was time shifted by this offset. All such $\tau$ ranged between -2 and 2 ms.

Normalization by input resistance and resting membrane potential

Neurons differed in resting potential and input resistance. To reduce the influence of these properties on classification by activity under perturbation, the response waveforms were normalized in one of two ways. The first method was by linearly transforming the response waveforms:

$$r_{xform}(t) = a + b \cdot r(t + c)$$

where $a$ is a current offset to compensate for differences in resting membrane potential, $b$ is an amplitude adjustment to compensate for differences in input resistance, and $c$ is a timing adjustment to compensate for any remaining timing errors not found in alignment. The linear transformation was performed before each comparison. One waveform was chosen as the reference, and the other was transformed with values of $a$, $b$, and $c$ which maximized the overlap between the two waveforms (Figure 53). The resulting normalized waveforms were designated the transformed response waveforms.

To determine the appropriate values of $a$, $b$, and $c$, these parameters were fit using a Nelder-Mead simplex (“amoeba”) optimizer (Nelder JA and Mead R, 1965), which performs a
gradient of parameter space until reaching a local minimum (Chapter 3 - Materials and Methods - Assessing pathfinding performance for a full description of this algorithm). The optimizer was implemented using the `nm_simplex` functions of the GNU Scientific Library (gnu.org) and distributed on the parallel computing cluster. The optimizer adjusted the transform parameters until the waveform distance ($D_w$) between the current response traces were minimized.

![Waveform normalized by linear transformation.](image)

*Figure 53.* Waveform normalized by linear transformation.

Voltage clamp response waveforms from two VD neurons (VD1, red; VD2, blue) are shown before (A) and after (B) linear transformation of the VD2 waveform to maximize overlap.

Another method used to normalize responses to voltage clamp perturbation and compensate for differences in input resistance and resting potential was to subtract the passive component of the response. A single-compartment passive model was implemented in C using the GNU scientific library’s Runge-Kutta integrator methods (www.gnu.org/gsl), and stimulated using the same perturbation used on the real neurons. The free parameters of this model included membrane capacitance ($c_m$), leak conductance ($g_{leak}$), and leak current reversal potential ($E_{leak}$). For each response waveform, the free parameters of the passive model were fit using a Nelder-
Mead simplex; these parameters were adjusted until the waveform distance between the passive model’s output and the real neuron’s output were minimized (Figure 54).

Figure 54. Waveform normalized by subtraction of passive response.

Response waveforms from two IC neurons (IC1, red; IC2, blue) are shown before (A) and after (B) subtraction of passive components. The passive component was calculated by fitting a single-compartment passive model to the real neuron’s response over a 20 s region of mostly passive activity, adjusting $c_m$, $g_{\text{leak}}$, and $E_{\text{leak}}$ using a Nelder-Mead simplex. This 10 s sample was taken from that region, so most of the neurons’ responses are passive.

Waveform distance was calculated only over a 20 s region of the pink noise perturbation wherein activation of voltage-dependent conductances, other than the H conductance, was minimal. The response of the fitted model over the entire perturbation was then subtracted from the original waveform. The results of these subtractions were designated the **residual active waveforms**. These waveforms consisted of the current flow due to the voltage-dependent conductances (minus than the mean $I_H$ that occurred during fitting) as well as any passive properties due to non-isopotentiality.
Low-pass filtering

Where noted, the response waveforms were low-pass filtered. This was achieved by performing a fast Fourier transform of the input waveform, removing all complex amplitude values above the corner frequency and performing the inverse transform. No attempt was made to prevent ripples associated with the sharp corner frequency.

Determining clamp quality

To determine the faithfulness of the voltage clamping, the waveform distance was calculated between the voltage waveforms from each pair of voltage clamp recordings. Note that these were the actual neuron membrane potentials read from the Axoclamp, not the command voltages. For each voltage clamp voltage waveform \( v_i \), the mean waveform distance between it and every other voltage waveform \( v_j \) was calculated to give a clamp error factor:

\[
e_t = \frac{1}{n-1} \sum_{j=1}^{n} D_W(v_i, v_j)
\]

A high error factor indicated that the voltage waveform differed to a large degree from many or most other voltage waveforms, whether due to poor clamp stability, noise, or other artifacts. A histogram was then made of these clamp error factors (Figure 55). A threshold was visually chosen at the beginning of the long tail, and waveforms with clamp error above this threshold were not used in clustering or analysis. A common cause of high error factor was inability to hold the neuron at the most depolarized potentials, possibly due to poor seal and high shunt current with the two-electrode impalement procedure.
Hierarchical clustering without probabilities

Neurons responses were classified using hierarchical clustering, in two different steps. The first clustering step was performed without any assignment of probability to clusters. Typically, hierarchical clustering begins with an observation matrix of \( k \) observations (regular current samples, in this case) made for each of \( j \) entities (waveforms) to be clustered. However, the length of the digitized waveforms (200 s total duration, sampled at 10 kHz, for a total of 2 million values per waveform) made clustering using such an observation matrix impractical. Instead, the waveform distances \( D_W(r_1, r_2) \) were calculated between every two response waveforms \( r_1 \) and \( r_2 \) in the set. This was either done using the transformed response waveforms which were normalized to compensate for differences in input resistance and resting potential, or using the residual active waveforms which were normalized by removing the passive component.

\[\text{Figure 55. Voltage clamp quality histogram}\]

For each voltage clamp voltage recording, mean waveform distance from every other voltage waveform was calculated to determine a clamp error factor. Histogram shows all such error factors, using logarithmic bin sizes, and the chosen threshold (dotted vertical line). The voltage recordings in the “long tail” typically contained artifacts such as clamp ringing, incomplete clamping at depolarized potentials, voltage offsets, noise, or transients.
of the response. The result was a **distance matrix** which associated every two waveforms $r_1$, $r_2$ with a distance $d$.

This distance matrix was then used as input to the clustering algorithm implemented in custom software written in Perl and C, utilizing the high-performance computing cluster. This algorithm started with every waveform constituting a singleton cluster, and with the distances between all singleton clusters known. It proceeded by repeatedly choosing the two closest clusters $c_1$, $c_2$ in the set, joining them together to become a new single cluster object $c_{1+2}$, and then calculating distances from this new object $c_{1+2}$ to every other object $c_k$. This process was repeated until all clusters had been joined together into a single cluster, forming a hierarchy (**Figure 56**).

Calculation of distances between a newly formed cluster and other clusters occurred using one of several standard **linkage methods**. When clustering objects with spatial coordinates, the choice of linkage methods is typically straightforward, as the geometric center of a cluster can be calculated, and distance between that cluster and others is thus the Euclidean distance from this center to the other objects. For non-spatial data however, such as waveform recordings, calculating distance between a newly formed cluster ($c_i, c_j$) and another cluster $c_k$ is not so straightforward. Several standard linkage methods exist for calculating these distances. The three used herein included complete linkage, average linkage (also called UPGMA, unweighted pair group method with arithmetic mean), and Ward’s method.
Complete linkage defines the distance between a newly formed cluster \((c_i, c_j)\) and a third cluster \(c_k\) as the maximum distance to all members of the new cluster, i.e.,

\[
d(c_{i+j}, c_k) = \max \left( d(c_i, c_k), d(c_j, c_k) \right)
\]

This has the effect of encouraging the clustering algorithm to form several small clusters first, before joining them together into a hierarchy. Average linkage defines the distance between a newly formed cluster \((c_i, c_j)\) and a third cluster \(c_k\) as the average of all distances within them:

Figure 56. Hierarchical clustering with complete and average linkage.

The first cluster is formed (A) with the closest objects \(a\) and \(b\), at a distance of 3, i.e., \(d(a, b)=3\). The distance from the new cluster \(ab\) to \(c\) is calculated using individual distances \(d(a, c)=5\) and \(d(b, c)=6.7\) (B). In complete linkage (C, left), \(d(ab, c) = \max(d(a, c), d(b, c))\), i.e., 6.7. In average linkage (C, right), \(d(ab, c)\) is the mean of all individual distances, i.e., \((5+6.7)/2 = 5.9\).

Distances among all objects are compared (D), and the next cluster is formed by joining the two closest objects (E). The third iteration would join all objects together.
$d(c_{i+j}, c_k) = \frac{|c_i|d(c_i, c_k) + |c_j|d(c_j, c_k)}{|c_i| + |c_j|}$

where $|c_i|$ is the number of elements in cluster $c_i$. UPGMA is thus the unweighted average of all distances of elements from one cluster to another. Ward’s method, which minimizes variance within a cluster (Ward JH, 1963), defines the distance between $(c_i, c_j)$ and $c_k$ as

$d(c_{i+j}, c_k) = \frac{(|c_i| + |c_j|)d(c_i, c_k) + (|c_j| + |c_k|)d(c_j, c_k) - |c_k|d(c_i, c_j)}{|c_i| + |c_j| + |c_k|}$

The results of the clustering were rendered as *dendrograms*, visual representations of the cluster hierarchy in which the length of a link between clusters represents the distance between those clusters.

**Hierarchical clustering with probabilities**

The previously described hierarchical clustering performed on the precalculated distance matrix could not provide statistical information about the validity of the clustering. The method typically used to calculate p-values to such clustering is *bootstrapping*. In bootstrapping, samples are taken with replacement from each vector in the original observation matrix to create a new matrix, and the clustering is performed on this new observation matrix. For example, if the observation matrix waveform consisted of $N = 1,000$ samples (observations) from $k = 100$ waveforms, a single bootstrapping iteration would begin by creating 100 new waveforms; each new waveform $r'_i$ would consist of $N$ samples randomly chosen with replacement from the original $N$ samples of $r_i$. The clustering is then repeated using the resampled observation matrix. Bootstrapping typically involves many iterations (10,000 is common). Due to the large number of samples per waveform (2 million), bootstrapping of the original observation matrix was not feasible.
To facilitate bootstrapping, each waveform to be clustered was low-pass filtered at 100 Hz, and then downsampled at 10 ms intervals, to produce a total of 20,000 observations (sampled current values) per waveform. The \texttt{pvclust} and \texttt{scaleboot} packages in the open source statistical computing language R (r-project.or) were then used to conduct multiscale bootstrapping and calculate p-values (Shimodaira, 2002; Shimodaira, 2004) using Euclidean distance and average linkage. Both Bootstrap Probability (BP) (Felsenstein J, 1985) and Approximately Unbiased (AU) (Shimodaira, 2002) p-values were calculated. Adjustments were made for non-smooth boundaries (Suzuki et al., 2008). In multiscale bootstrapping, the resampling size $N_i$ is varied in $k$ steps, e.g., from $0.5N$ to $1.4N$. For each step, bootstrapping occurs as usual, but with $N_i$ samples taken instead of the original $N$. The number of times $C_i$ a given cluster appears in the $i$th resampling size is recorded, and the AU p-value for the cluster is calculated from the change in $C_i$ as $N_k$ is varied (Shimodaira, 2002).

Relative resampling sizes of 0.11, 0.16, 0.23, 0.33, 0.48, 0.69, 1, 1.44, 2.08, 3, 4.33, 6.24 and 9 were used (i.e., $N_1 = 0.11 \cdot N$, $N_2 = 0.16 \cdot N$, …), where $N = 20,000$ current samples from the downsampled waveform. Bootstrapping was repeated for 20,000 replications per resampling size. Fitting diagnostics provided by the statistical package were visually examined to verify that replication size was sufficient. Computation was distributed across the high-performance computing cluster using the \texttt{snow} and \texttt{Rmpi} packages of R. The results of the clustering were visualized as dendrograms showing linkage and BP and AU p-values.

\textit{Segment root mean square current}

The optimized perturbation contained different segments, each of which was evolved to elicit a particular voltage-dependent current. To determine whether different pyloric neuron types (AB, IC, VD, LP, PD, or PY) responded in distinctive ways to these different segments, the current response waveforms were split into eight segments corresponding to the eight
perturbation segments. Within each segment of a response waveform $r$, the root mean square (RMS) current was calculated:

$$I_i = \frac{1}{t_2 - t_1} \sqrt{\int_{t_1}^{t_2} r(t)^2 \, dt}$$

where $t_1$ and $t_2$ are the start and end times of the segment, and $i = 1 .. 8$. Three ANOVA models were then made to account for variance in RMS current by neuron type, by segment, or by both neuron type and segment (two-factor). The two-factor model was compared against each one-factor model to determine whether the former accounted for significantly more variance in RMS current, again using an ANOVA.

*High performance computing*

Where feasible, software was parallelized and executed on a high performance computing cluster of 8 nodes, 8 cores per node, 2 threads per core. Hardware was sourced from Advanced Clustering Technologies, Inc. Task distribution and message passing were achieved using OpenMPI (open-mpi.org) and custom infrastructure written in C.
Results

Neurons responded well to the optimized perturbation protocols, generally exhibiting stable resting potentials throughout the experiments. Voltage-dependent conductances were clearly visible in the responses to the voltage clamp perturbations, with some neuron types often showing visibly distinct characteristic responses (Figure 57), e.g., IC neurons were generally passive, PD neurons had large, slowly-deactivating currents in response to the initial depolarization, etc. Responses during the second repeat of the conductance-optimized perturbations were generally visibly similar to those of the first.

Figure 57. Response of PD, IC, and VD neurons to **cp1** composite perturbation.

Different neuron types responded in characteristic ways to the perturbation, though, these were generally differences in degree rather than absence or presence. For example, PD neurons had large exponentially decaying responses to the first segment (red), ICs had larger responses in the second segment (green), and VDs had linearly decaying current responses to the first segment and rapidly decaying responses to the eighth segment (blue).
Current clamp perturbations were also applied. Although they were not used in this analysis, visualization of the membrane potential responses to current injection revealed slow depolarizations after current perturbation in some neurons, and slow hyperpolarizations after perturbation in others (*Figure 58*). The slow depolarizations were likely due to the hyperpolarization-activated sag current $I_{H}$, but the decay time of the current did not match the dynamics of this current in the Prinz model. The cause of the slow hyperpolarization was unknown. It did not match the dynamics of any current in the model, and was both too slow and too large to be capacitive discharge.

*Figure 58*. Slow hyperpolarizations and depolarizations after current clamp perturbation.

In response to the same current clamp perturbation, a PY neuron (A) exhibits a slow hyperpolarization of membrane potential after current stimulation is stopped, whereas a PD neuron (B) exhibits a slow depolarization. The latter is due to $I_{H}$ though the dynamics do not match the $I_{H}$ current in the neuron model used to develop the perturbations. The cause of the slow hyperpolarization observed in some PY neurons is unknown. Dashed lines show resting potential in each neuron.
Residual active waveforms

From each current response waveform obtained from the voltage clamp perturbations, a **residual active waveform** was constructed, by accounting for as much of the passive electrical activity of the neuron as possible and subtracting that passive activity from the waveform trace (Materials and Methods - Normalization by subtraction of passive responses). The resulting residual waveforms continued to demonstrate evidence of voltage-dependent conductances and characteristic responses by neuron type (Figure 59).

![Figure 59](image-url)

**Figure 59.** Residual active waveforms from PD, IC, and VD neuron.

The passive component of each neuron response waveform was removed by fitting a passive model to the response over a region with low activation of voltage-dependent conductances, then subtracting the passive model’s activity from the waveform. The three different neurons continue to show type-specific responses to the perturbation. Compare Figure 57. These residual active waveforms were used where noted.
**Waveform distances within and across neuron types**

The current response waveforms were then evaluated to determine whether responses from neurons of a given type, e.g., PDs, were more similar to responses of the same type than to responses of other types. Only the first perturbation made to each neuron was used, and only if its voltage clamp error factor was below the threshold. The AB neuron was excluded from this analysis as only one AB neuron was successfully voltage clamped. Waveform distances were then calculated among the 43 remaining waveforms (*Table 11*), with linear transformation to compensate for differences in input resistance or resting potential (*Materials and Methods - Normalization by input resistance and resting membrane potential*).

**Table 11.**

Mean ± standard deviation of waveform distances between neuron types

<table>
<thead>
<tr>
<th></th>
<th>IC</th>
<th>LP</th>
<th>PD</th>
<th>PY</th>
<th>VD</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>2.33 ± 0.67</td>
<td>4.97 ± 1.58</td>
<td>7.39 ± 2.08</td>
<td>6.20 ± 3.95</td>
<td>6.33 ± 1.75</td>
</tr>
<tr>
<td>LP</td>
<td>4.97 ± 1.58</td>
<td>6.87 ± 5.32</td>
<td>12.70 ± 4.32</td>
<td>8.21 ± 5.65</td>
<td>9.06 ± 3.80</td>
</tr>
<tr>
<td>PD</td>
<td>7.39 ± 2.08</td>
<td>12.70 ± 4.32</td>
<td>5.32 ± 1.64</td>
<td>13.47 ± 4.29</td>
<td>7.04 ± 1.66</td>
</tr>
<tr>
<td>PY</td>
<td>6.20 ± 3.95</td>
<td>8.21 ± 5.65</td>
<td>13.47 ± 4.29</td>
<td>9.38 ± 6.17</td>
<td>10.78 ± 4.08</td>
</tr>
<tr>
<td>VD</td>
<td>6.33 ± 1.75</td>
<td>9.06 ± 3.80</td>
<td>7.04 ± 1.66</td>
<td>10.78 ± 4.08</td>
<td>4.07 ± 1.05</td>
</tr>
</tbody>
</table>

An ANOVA and Tukey’s Honest Significant Differences post-hoc test were conducted which identified significant differences among groups. ICs were more like ICs than any other type (p<0.01), and more like LPs than PDs (p<0.0001). PDs were more like PDs than any other
type (p<0.0001), and more like VDs or ICs than like LPs or PYs (p<0.0001). VDs were more
like VDs than other type (p<0.01), and more like PDs or ICs than LPs or PYs (p<0.001). Among
LPs and PYs, however, neurons were not more like their own type than all other types. Instead,
both LPs and PYs were more like ICs than PDs, PYs, or VDs, and more like VDs than PDs (all
p<0.001). Similar results were obtained when the residual active waveforms were used for
distance calculations. The residual active waveforms from ICs, PDs, and VDs were significantly
closer, in waveform distance, to waveforms of their own type than to any other type (p<0.001).

Hierarchical clustering of transformed waveforms without probabilities

Due to the recording length (approximately 200 s) and sampling rate (10kHz), the data
size made traditional clustering on a full observation matrix infeasible. Instead, waveform
distances were precalculated among all 43 transformed response waveforms, and assembled into
a distance matrix. Clustering was then performed and dendrograms created using Ward’s method
(Figure 60), average linkage (Figure 61), and complete linkage (Figure 62). Results were similar
in all three dendrograms. PDs clustered together, in a group which also included the VDs as one
or two subclusters. ICs generally clustered together in a group which including the AB; the
exception was a single IC in average linkage clustering with a PY/LP group. PYs and LPs
clustered in one or more mixed groups. The tendency of VDs, PDs, and ICs to cluster among
their own type was stable regardless of linkage, whereas PY and LP clusters were less stable.

To further assess the stability of the clustering, an additional 9 recording were selected by
increasing the voltage clamp error threshold from 115 to 150. A distance matrix was made from
the 52 waveforms, and clustering was repeated using the three linkage methods (Ward’s method
is shown in Figure 63). Cluster integrity was maintained for VD, PD, and IC neurons. A group
of three LPs (LP2, LP13, and LP15) consistently appeared regardless of linkage, but otherwise,
PYs and LPs did not stably cluster.
Figure 60. Clustering of 43 transformed response waveforms with Ward’s method linkage.

Ward’s method minimizes variance within clusters. Text labels are colored according to neuron type (red: PY; green: PD; blue: VD; cyan: LP; magenta: IC; brown: AB). Horizontal distance to connecting linkage represents waveform distance between neuron recordings. Line color represents cluster membership.
Figure 61. Clustering of 43 transformed response waveforms with average linkage.

Average (UPGMA) linkage defines the distance between two clusters as the mean of all the distances between members of one cluster and another. Text label color, line length, and line color as in Figure 60.
Figure 62. Clustering of 43 transformed response waveforms with complete linkage.

Complete linkage defines the distance between two clusters as the maximum distance between members of one cluster and another, thus preferentially creating more, smaller clusters at lower levels of the hierarchy. Text label color, line length, and line color as in Figure 60.
Figure 63. Clustering of 52 transformed response waveforms with Ward’s method linkage.

An additional 9 recordings were included in the original 43 by increasing the voltage clamp error threshold to 150. Cluster stability was retained. Text label color, line length, and line color as in Figure 60.
Hierarchical clustering of residual active waveforms without probabilities

The transformed response waveforms were normalized by linear translation, with one clamp current waveform amplified or attenuated and offset to maximize overlap with the other. This was done to compensate for differences in input resistance or resting membrane potential between neurons. However, scaling the entire waveform also scaled the portion of the waveform due to active conductances. Thus, a second normalization method, removal of the passive electrical component of the waveform (Materials and Methods - Normalization by subtraction of passive responses), was also used, to create residual active waveforms from each original current clamp recording. The hierarchical clustering process was repeated using the 43 residual active waveforms with voltage clamp error below threshold, again using Ward’s method (Figure 64), average linkage (Figure 65), and complete linkage (Figure 66). Results were similar to those obtained from clustering the transformed response waveforms. With few exceptions, VDs clustered with VDs and PDs with PDs. ICs generally clustered with other ICs and the AB, with occasional inclusions of PYs. The VD and PD groups were more separate in the residual active waveform dendrograms than in the transformed response waveform dendrograms. Clustering with a threshold of 150 (53 recordings) produced similar results.
Figure 64. Clustering of 43 residual active waveforms with Ward’s method linkage

The passive electrical component of each recording was subtracted, after fitting a passive neuron model using the voltage clamp perturbation and the current response. Clustering results were similar to the corresponding results from the original 43 waveforms (Figure 60).
Figure 65. Clustering of 43 residual active waveforms with average linkage.

Clustering results were similar to the corresponding results from the original 43 waveforms (Figure 61).
Figure 66. Clustering of 43 residual active waveforms with complete linkage

Clustering results were similar to the corresponding results from the original 43 waveforms (Figure 62).
Hierarchical clustering with probabilities

Though visually compelling, the previously described dendrograms did not provide statistical confidence about the validity of the clustering. Bootstrapping from the full observation matrix of 43 waveforms of 2 million samples each, was infeasible with existing computational resources. However, an initial investigation with low-pass filtered waveforms suggested that an observation matrix could be formed from downsampled waveforms. The residual active waveforms were thus low-pass filtered at 100 Hz and downsampled at 10 ms sample intervals (20000 samples per waveform). Clustering with multiscale bootstrapping was then performed using the observation matrix (Materials and Methods - Hierarchical clustering with probabilities) (Suzuki et al., 2008; Shimodaira, 2004). The clustering algorithm calculated p-values using both Bootstrap Probability (BP) and Approximately Unbiased (AU) methods. Clustering was repeated, and dendrograms were visualized, using the three linkage methods: Ward’s method (Figure 64), average linkage (Figure 65), and complete linkage (Figure 66). For all three linkages, significant clusters of PD and VD neurons were identified which contained many or all members of their type. In two of three linkages, there were significant clusters of three ICs, adjacent to clusters of two ICs and the AB. Three PYs (PY10, PY22, and PY26) significantly clustered together in two of the three linkages, but otherwise, PYs and LPs were inconsistently distributed in numerous small clusters.
Figure 67. Clustering with bootstrapping of 43 residual active waveforms with Ward’s method linkage.

Bootstrapping was performed to calculate cluster probabilities (*: p<0.05; **: p<0.01). Both Bootstrap Probability (green asterisks) and Approximately Unbiased (red asterisks) methods were used to calculate p-values. Boxes were drawn around each largest significant (AU or BP p<0.05) cluster whose subclusters were all significant (red: heterogeneous clusters; green: homogeneous clusters). Within heterogeneous clusters, significant homogeneous clusters containing three or more members were also identified (blue boxes).
Figure 68. Clustering with bootstrapping of 43 residual active waveforms with average linkage.

Asterisks indicate p-values (green: BP; red: AU; *: p<0.05; **: p<0.01). Boxes were drawn as in Figure 67.
Segment root mean square current

The 43 transformed response waveforms were split into segments, each corresponding to a segment of the voltage clamp perturbation optimized for a particular conductance. As the conductance-optimized perturbation section was repeated in the \textbf{cp1} composite perturbation, this produced a total of 8 segments, for $I_{\text{KCa}}$, $I_{\text{Na}}$, $I_{\text{Kd}}$, $I_{\text{NaP}}$, $I_{\text{CaS}}$, $I_{\text{CaT}}$, $I_{\text{H}}$, and $I_{\text{A}}$, with two replications per segment. Total root mean square current was calculated for each segment. This process was then repeated for the residual active waveforms. Current in the various neuron types appeared similar for both replications of a segment, except for the first segment. Different neuron types did appear to have different characteristic responses to each segment (Figure 70). For example, in

\textbf{Figure 69.} Clustering with bootstrapping of 43 residual active waveforms with complete linkage. Asterisks indicate p-values (green: BP; red: AU; *: p<0.05; **: p<0.01). Boxes were drawn as in \textbf{Figure 67}.
the third segment, originally optimized to elicit $I_{Kd}$, LP and PY neurons responded strongly, whereas IC, PD, and VD neurons did not. In the fourth segment, optimized for $I_{NaP}$, LP, PY, PD, and VD neurons all responded. Response patterns appeared similar for both the transformed and residual active waveforms.

Figure 70. Root mean square current per segment per neuron type.

The $cp1$ composite perturbation contained two replications of a conductance-optimized perturbation series; each segment in the perturbation was evolved to optimally activate a given voltage-dependent conductance ($x$ axis). The RMS current over each segment was measured for the 43 transformed response waveforms (A) and corresponding residual active waveforms (B), for each neuron type, and for both replications. Error bars show mean ± standard deviation of RMS current. A comparison of single-factor (neuron type or segment) and two-factor (type and segment) ANOVA models confirmed that different neuron types had characteristic responses to the different segments of the perturbation.
To confirm these differences, single-factor and two-factor ANOVA models were made using neuron type and segment number, and the two-factor model was compared against the single-factor models. A model including both neuron type and segment was significantly better (p<0.001) than a model including only type or only segment. This was true for both the transformed waveforms (type+segment vs type, F=78; type+segment vs segment; F=42) and for the residual active waveforms (type+segment vs type, F=65; type+segment vs segment, F=57).

Time stability

To assess whether drift had occurred in neuron responses over time, recordings were assembled for those neurons which had been perturbed multiple times. This included a total of 49 neurons (PD=6, VD=8, IC=7, LP=10, PY=16, AB=2). For every recording taken, beginning with the second, waveform distance was calculated between that recording and the first recording taken in that neuron. Linear transformation was used to compensate for changes in membrane potential or input resistance. Waveform distance from first perturbation was plotted against time, in seconds, since impalement (Figure 71). A linear regression model was constructed on the waveform distances and impalement times. The resulting model ($D_w = 2.74\pm1.21 + 0.00096\pm0.0003\cdot t$), was significant at p<0.002 ($R^2 = 0.0645$, DF=129); the slope was significant at p <0.002 ($t$ in seconds). This process was repeated for the residual active response waveforms, over the same neurons. Again, the regression model ($D_w = 4.25\pm1.74 + 0.00128\pm0.00044\cdot t$), was significant at p < 0.004 ($R^2 = 0.0545$); the slope was significant at p < 0.005.
**Figure 71.** Waveform distance between first and subsequent transformed response waveforms in neurons subjected to multiple perturbations.

For neurons subjected to three or more cp1 perturbation repeats, waveform distance ($D_W$) was calculated between the first response, and each subsequent response. $D_W$ was plotted versus time since impalement. $D_W$ generally increased over time for all neuron types. A regression model confirmed this was statistically significant in both the transformed waveforms (shown) and residual active waveforms.
Discussion

During the perturbations, it was discovered that the Prinz model was not necessarily a good fit for recordings from all *Panulirus interruptus* pyloric neuron types. For example, in current clamp, PD neurons often exhibited a substantial putative $I_{hi}$ current whose dynamics did not match the predictions of the Prinz model (Prinz et al., 2003), and PY neurons exhibited a slow hyperpolarization after removal of depolarizing stimulation (*Figure 58*). The cause of the latter is not known. As such, it was expected that a perturbation protocol optimized for a neuron model may not yield good performance in neurons which did not fit this model.

Initial statistical tests, however, confirmed that waveform distances among different neuron types did differ significantly, and that furthermore, PD, VD, and IC neurons were more similar to neurons of their own type than other types. This pattern was repeated in subsequent stages of the investigation.

The conductance-optimized perturbation was empirically designed to maximize the activity of the voltage-dependent conductances. Real neurons, however, also contain a leak conductance, and may additionally contain a soma shunt as a result of impalement damage. To reduce the influence of leak or shunt currents on the results, the neuron recordings were normalized in one of two ways. The first method was simple scaling and linear translation of the waveforms against an arbitrary reference (the *transformed response waveforms*); the second was fitting a passive model and subtracting the passive electrical response (the *residual active waveforms*). Throughout this research, the results obtained using the two methods were similar, suggesting that the normalization method did not introduce artifacts into the results.

An initial cluster analysis was performed using hierarchical clustering on the distance matrix made from first perturbation recordings. Note that these distances were simply the Euclidean waveform distances over the entire time-domain waveform, unfiltered, and sampled at
a very high rate (10kHz), and contained many (~ 2 million) current samples. No attempt was made to identify relevant features *a priori* or otherwise process the waveforms so as to reduce the number of dimensions, as has been conducted in other, k-medioids based cluster analysis of stomatogastric neuron responses (Brookings et al., 2012). Instead, it was hypothesized that the responses of the neurons would be sufficiently information-dense for the cluster analysis to partially or fully recapitulate the nominal types (PD, LP, PY, etc.). The initial results provided visual support for this hypothesis, with robust clusters of PDs, VDs, and ICs, the VDs appearing as a subset of the PDs, and the ICs including the ABs. LPs and PYs clustered in several disjoint groups. This taxonomy persisted when linkage method was changed, when additional recordings were included, or when the residual active waveforms were used.

While the robustness of the taxonomy was compelling, these dendrograms did not offer statistical confidence. Unfortunately, the usual method for statistically validating hierarchical clustering, bootstrapping, required a prohibitively large observation matrix. Downsampling versions of the residual active waveforms, low-pass-filtered at 100Hz, were used instead. Despite the considerable reduction in number of samples – the downsampled waveforms were only 1% of the size of the originals – and the loss of high-frequency components, clustering with multiscale bootstrapping identified several statistically significant clusters of VD, PD, and IC neurons. Again, VDs typically appeared as a subtaxon or sister taxon of PDs, and ICs appeared with the ABs. This taxonomy persisted across changes in linkage method.

The specific segments of the optimized perturbation did appear to stimulate different neuron types differently. This was visually suggested (*Figure 57*), and was confirmed statistically by comparing one and two factor models. Note that it is not necessarily the case that the segment of a perturbation which was evolved for a given current in the Prinz model will elicit the same current in a real neuron. While PD neurons may respond especially well to the
segments evolved for $I_A$ and $I_{NaP}$, but not nearly as well to the $I_{Kd}$ segment, it could be that the “$I_{Kd}$” segment is actually eliciting $I_{KCa}$, or that the “$I_{NaP}$” segment is eliciting $I_{CaS}$.

The lack of distinction between LP and PY neurons is not necessarily surprising. During experiments it was noted that the PY neurons, which were not especially robust to begin with, appeared to lose much of their character rapidly after impalement, becoming increasingly passive. Visual review of the LP neuron responses did not reveal any particularly characteristic responses; if they exist, they may be too subtle to be detected by the methods used herein. Repeating the electrophysiology with more stringent criteria for including or excluding PY and LP neurons could provide clarity. It has also been suggested that PYs may exist in “early” and “late” types, based upon timing within the ganglion, or may exist in a continuum between these types (Hartline and Graubard, 1992). If differences in intrinsic properties underlie these timing differences, then the lack of clear clustering of PY neurons may be a result; for example, “early” PYs could cluster closer to the ICs, and “late” PYs closer to LPs. Furthermore, cluster analysis relies upon the samples existing in discrete groups. If PY neurons exist in a continuum from early to late forms, then finding discrete groups may be impossible. Another possibility is that the ion channels in the PY and LP neurons are largely inaccessible to voltage clamp, if for example these neurons are appreciably non-isopotential.

Methods which extract relevant features of the raw waveform data, such as Fourier or wavelet transform, may well produce stronger results. However, the fact that three of the five readily accessible neuron types are distinguishable through relatively simple analytical methods demonstrates that the cp1 composite perturbation protocol is effective at eliciting active behavior in a meaningful way that recapitulates previously identified type. This confirms the ability of the cp1 perturbation to separate neurons based on intrinsic properties, and further suggests that, at least for the three robustly clustering neuron types, the intrinsic properties of these neurons may
vary less within the groups than between them. The robustness of these results also suggests that sufficient voltage-activated current may be elicited by the \texttt{cp1} perturbation for heuristic methods such as evolution strategies to determine parameters which reproduce activity of individual neurons, assuming the underlying model is appropriate.

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Disclosures

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CHAPTER 5: CONTAMINATION OF CURRENT-CLAMP MEASUREMENT OF NEURON CAPACITANCE BY VOLTAGE-DEPENDENT PHENOMENA

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Author Contributions

This research stems from a long-running project in the S.L.H. lab devoted to building quantitatively accurate lobster pyloric neuron models. Prior data from multiple workers in the S.L.H lab showed that a major roadblock in this project is the lack of accurate neuron capacitance data. W.E.W. independently chose to measure pyloric neuron capacitance accurately, noted that naïve application of prior techniques gave capacitance values vastly different from neuron tracing data already present in the lab, and decided that performing the measurements at multiple membrane potentials might help resolve this discrepancy. W.E.W and S.L.H. contributed to subsequent research design and interpreted experimental results. W.E.W. performed all experiments, wrote all custom software, and analyzed all data. W.E.W. prepared initial versions of figures, S.L.H. prepared final versions. W.E.W. drafted manuscript; W.E.W. and S.L.H. edited and revised manuscript and approved final version.

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Abstract

Measuring neuron capacitance is important for morphological description, conductance characterization, and neuron modeling. One method to estimate capacitance is to inject current pulses into a neuron and fit the resulting changes in membrane potential with multiple exponentials; if the neuron is purely passive, the amplitude and time constant of the slowest exponential give neuron capacitance (Major et al., 1993). Golowasch et al. (Golowasch et al., 2009) have shown that this is the best method for measuring the capacitance of non-isopotential (i.e., most) neurons. However, prior work has not tested for, nor examined how much error would be introduced by, slow voltage-dependent phenomena possibly present at the membrane potentials typically used in such work. We investigated this issue in lobster (*Panulirus interruptus*) stomatogastric neurons by performing current clamp based capacitance measurements at multiple membrane potentials. A slow, voltage-dependent phenomenon consistent with residual voltage-dependent conductances was present at all tested membrane potentials (-95 to -35 mV). This phenomenon was the slowest component of the neuron’s voltage response, and failure to recognize and exclude it would lead to capacitance overestimates of several hundred-fold. Most methods of estimating capacitance depend on the absence of voltage-dependent phenomena. Our demonstration that such phenomena make non-negligible contributions to neuron responses even at well-hyperpolarized membrane potentials highlights the critical importance of checking for such phenomena in all work measuring neuron capacitance. We show here how to identify such phenomena and minimize their contaminating influence.

Keywords: stomatogastric, lobster, *Panulirus interruptus*
Introduction

Capacitance is a defining characteristic of neuron electrical properties. Because membrane specific capacitance varies little across neurons, typically being 0.9 µF/cm² (Gentet et al., 2000), neuron capacitance also estimates neuron surface area. Accurately measuring membrane capacitance is therefore important in studying neuron development and morphology, characterizing neuron conductances, and neuron modeling. With respect to modeling, membrane currents change membrane voltage by acting through membrane capacitance. Model membrane potential dynamics thus critically depend on capacitance. Simply stated, no matter how accurate the description of neuron currents, without an accurate value of capacitance, neuron models give incorrect activity. In a bursting pyloric model neuron (Figure 72A), increasing capacitance two-fold increased inter-burst interval and decreased burst duration (Figure 72B), and a ten-fold increase sharply attenuated spike amplitude and further decreased burst duration (Figure 72C).

Accurate measurement of neuron capacitance is thus clearly required for accurate neuron modeling. Multiple electrical techniques for neuron capacitance measurement have been described. Capacitance can be measured in voltage clamp by integrating or fitting the currents that occur with step or ramp changes in voltage (Golowasch et al., 2009; Haedo and Golowasch, 2006; Iwasaki et al., 2008; Khorkova and Golowasch, 2007; Pineda et al., 2008; Royeck et al., 2008) or in current clamp by fitting the voltage responses to step changes in current (Arsenault et al., 2011; Firestein and Werblin, 1987; Liao and Lee, 2011; Major et al., 1993; Roth and Häusser, 2001; Szabo et al., 2010).

Current clamp techniques are most accurate for neurons with extended morphologies or non-uniform conductance distributions (non-isopotential neurons) (Golowasch et al., 2009). In these neurons the voltage response is typically fit by a sum of exponentials. In non-isopotential neurons with only passive (non-voltage dependent) electrical properties, and in which the
dendritic compartment dominates the neuron’s response (see Figure 3 of Golowasch et al., 2009), the slowest of these terms can be used to estimate neuron capacitance and resistance. The faster terms arise from current flow through the neuron’s branches (Holmes et al., 1992; Major et al., 1993; Rall, 1969) and can be used to estimate neuron electrotonic length (Rall, 1977).

![Figure 72](image)

*Figure 72.* Altering neuron capacitance changes neuron activity.

Doubling (B) and increasing capacitance ten-fold (C) changes the cycle period, burst duration, and amplitude of a model neuron (A). A common pyloric neuron model (Prinz et al., 2003) was used, having conductance $\bar{g}$ parameter values $5$ (Ca$_T$), $4$ (Ca$_S$), $40$ (A), $5$ (K$_{Ca}$), $125$ (K$_d$), $0.01$ (H), $200$ (Na) (all values in mS/cm$^2$) and base capacitance $0.628$ nF.

A potential difficulty with the current clamp technique is the presence of voltage-dependent phenomena with dynamics slower than the neuron’s passive dynamics (Taylor, 2011), in which case the slowest term arises from the dynamics of the slow phenomena, not from neuron capacitance and passive resistance. Since passive charging curves change monotonically, slow phenomena that cause voltage changes that oppose the neuron charge curve, e.g., $I_g$-induced voltage sags, are easily identifiable (Major et al., 1993; Rose and Dagum, 1988). However, for
slow voltage-dependent phenomena that cause voltage changes with the same sign as the charging curve, it is not obvious that the slow term does not arise from neuron passive properties, and thus should not be used to estimate neuron capacitance.

Since true neuron capacitance should not vary with membrane potential, one method to identify voltage-dependent phenomena is to perform current steps at multiple membrane potentials. We have performed such experiments on pyloric neurons of the lobster (*Panulirus interruptus*) stomatogastric ganglion and show that the voltage responses are indeed contaminated by a slow voltage-dependent process. Misidentifying this slowest term in the multi-exponential fits as arising from passive properties would over-estimate neuron capacitance many hundred-fold. To our knowledge, previous current clamp measurement of neuron capacitance, regardless of species, has not been performed at multiple membrane potentials. The presence of slow voltage dependent phenomena has therefore not been tested for in any of this work and it may thus be compromised by unrecognized slow voltage-dependent phenomena.

Materials and Methods

*Electrophysiology*

*P. interruptus* were obtained from Marinus Scientific (Newport Beach, CA) and maintained in aquaria at 10-13˚C with circulating artificial seawater. Dissections were standard (Selverston et al., 1976). Preparations were perfused with 10-13˚C *P. interruptus* saline (479 NaCl, 12.9 KCl, 13.7 CaCl₂, 3.9 Na₂SO₄, 10 MgSO₄, 10.9 dextrose, 11.1 Tris, 5.1 maleic acid, all in mM; pH adjusted to 7.4-7.6; Fisher Scientific) throughout the experiment. Extracellular recordings from the lateral and medial ventricular nerves were made with an A-M Systems differential amplifier using monopolar stainless steel electrodes insulated with petroleum jelly. Intracellular recordings were made with an Axoclamp 2B and sharp glass microelectrodes pulled on a P-87 Flaming/Brown micropipette puller (Sutter Instruments) and filled with 2.5 M KAc, 20
mM KCl (resistance 10-20 MΩ). Lateral Pyloric (LP) and Pyloric (PY) neurons were identified by comparing intracellular and extracellular activities.

Following neuron identification, preparations were bathed in *P. interruptus* saline containing 0.1 µM tetrodotoxin, 10 µM picrotoxin, and 5 mM CsCl to block fast sodium spikes, synapses, and hyperpolarization-activated $I_{hi}$ current. LP or PY neurons were impaled with two glass microelectrodes, one to measure voltage and the other to pass current. The Axoclamp 2B amplifier was used in bridge mode. Data were digitized at 5 kHz with a Cambridge Electronic Design 1401plus intracellular amplifier and processed with Spike2 software. Square wave pulses were generated using custom-written Spike2 scripts.

*Setting current pulse amplitude*

Current pulse amplitude, $I_{pulse}$, was set by injecting bias current to hold the neuron at -50 mV for 60 s, and then injecting 1 s on 1 s off square wave current pulses of sufficient amplitude to hyperpolarize the neuron to -60 mV. This $I_{pulse}$ was used throughout the experiment, and was not adjusted to compensate for changes in neuron input resistance ($R_N$) that might have occurred during the experiment. Not compensating for $R_N$ changes did not affect analysis validity as $R_N$ was not used in determining capacitance. These calculations do depend on membrane potential. The data were therefore always plotted and analyzed using measured membrane potential.

*Current stairsteps*

In five PY and four LP neurons, four or six different membrane potentials ($V_{ssc}$, the steady-state membrane voltage at a given bias current level) were selected: -95, -80, -65, -50, -42.5, -35 mV or -80, -70, -60, -50 mV. Each neuron was held at the first $V_{ssc}$ for at least 60 s, adjusting bias current as necessary. Square wave pulses of amplitude $I_{pulse}$ and 0.5 Hz frequency were then injected for 60 s. Bias current was then changed to reach the next $V_{ssc}$ and the process repeated (*Figure 73*; the inset shows an expanded version of a few current pulses at one $V_{ssc}$; note
that input resistance had decreased when this recording was made so current pulses did not produce a 10 mV deflection). The stai-step was repeated for 2-4 cycles.

**Figure 73.** Response of neuron LP2 to current-clamp stai-step protocol

Bias current was applied to reach and maintain a nominal membrane potential of -100 mV for 60 s, followed by 60 s of a 0.5 Hz fixed amplitude square wave pulse train added to the bias current (the actual steady-state potential achieved was used in all calculations). This process was repeated at six increasingly depolarized potentials. In this experiment the entire stai-step was repeated for two additional cycles. The inset shows three cycles of the neuron's response during the pulse train at -50 mV. Pulse train response amplitude was greater at -95 mV than at -50 mV (the membrane potential at which capacitance measurements are typically made (Golowasch et al., 2009)), indicating that input resistance ($R_N$) was greater at -95 mV.

**Determining mean voltage responses to the current pulses**

Waveform data were exported from Spike2 and processed using custom software written in Perl and C. Current clamp based methods for estimating capacitance depend critically on
accurate measurements of current pulse amplitude and timing. To maximize accuracy we therefore determined these two parameters algorithmically. Mean current pulse on and off amplitudes were determined from the current trace by constructing a kernel density estimator of the current values and finding the two maxima thereof. Pulse timing was recovered by adjusting the phase of a mathematically generated square wave of matching amplitude and frequency until maximum overlap with the current trace was achieved. This technique was used because it produced more accurate results than thresholding in preliminary tests. This timing information was used to find the start and end times of the membrane voltage response to each current step. The voltage responses to the first five current pulses were discarded to exclude any effects of slow process equilibration (see Results). All subsequent cycles were averaged to improve signal/noise ratio.

Expontential fitting and coefficient calculation

Hyperpolarizing and relaxing phases (each 1 s long) of each pulse train’s mean voltage response waveform from \( t = 0 \) to 990 ms were fit (Figure 75) with a sum of exponentials model

\[
V_m(t) = V_{\text{final}} + \sum_{i=0}^{n} A_i e^{-t/\tau_i}
\]

using software implementing a transform method (DISCRETE) incorporating the standard deviations of the mean voltage responses (Provencher and Vogel, 1980). The DISCRETE program selected the best number of exponential terms using a Fischer’s F test \((p=0.05)\) with Beale’s nonlinearity correction; at most three terms were permitted. If the standard error of any of the \( V_{\text{final}}, A_i, \) or \( \tau_i \) in a three-term fit exceeded 10%, two-term fits were used, which in all cases gave acceptable \( V_{\text{final}}, A_i, \) and \( \tau_i \) errors. Custom software written in Perl was used to make the \( A_i \) and \( V_{\text{final}} \) values for positive current steps (the relaxing phase of the current pulses) comparable to the \( A_i \) and \( V_{\text{final}} \) values for negative current steps. Resistive coefficients were calculated as
\[ R_i = A_i / I_{pulse} \] for each term. Capacitive coefficients were calculated as \( C_i = \tau_i / R_i \) (of which only one corresponds to neuron capacitance; see Introduction).

**Input resistance determination**

\( R_N \) was calculated for each pulse by dividing the amplitude of the membrane potential change at the end of each phase of the current pulse by \( I_{pulse} \). These \( R_N \) were then averaged to obtain a mean \( R_N \) for each pulse train at each nominal \( V_{ssc} \).

**Statistical analyses**

Means were compared with Student’s \( t \)-tests. Linear regressions were performed with the Perl Statistics::Regression package. Outliers were detected by calculating Cook’s distance \( D_i \) on the regression model (Bollen and Jackman, 1990). Where \( D_i \) exceeded \( 4/N \) (\( N \) = number of observations), the corresponding waveforms were visually examined and recordings with obvious noise or artifacts were rejected. When \( V_{ssc} \) was used in linear regressions, the mean membrane potential at the end of the current-off phase was used to determine \( V_{ssc} \).

**NEURON models**

To test the effect of a slow, depolarization-activated conductance on capacitance measurements (see Results) under completely defined conditions, we implemented a voltage-dependent current \( I_{sr} \) in NEURON as follows:

\[
I_{sr} = \bar{g}_{sr} \cdot m \cdot (V_m - E_{sr})
\]

\[
dm/dt = (m_\infty - m) / \tau_m
\]

\[
m_\infty = m_0 + \frac{1 - m_0}{1 + e^{-(V_m - V_{1/2})/k}}
\]

with \( \tau_m \), the time constant of the activation gate, fixed at 0.2 s (the typical \( \tau \) of the slowest exponential component). Both isopotential and non-isopotential models were constructed. The isopotential model comprised a single compartment with \( I_{leak} \) and \( I_{sr} \) currents. \( g_{leak}, \bar{g}_{sr}, V_{1/2}, k, \)
m₀, E₀, Eₗₑᵃᵏ, and Cₘ (membrane specific capacitance per unit area) were adjusted during fitting (see below). The non-isopotential “ball and stick” model comprised two isopotential compartments (soma and distal) containing Iₗₑᵃᵏ and Iₛᵣ separated by a 10 µm diameter cable containing only Iₗₑᵃᵏ. Soma $\bar{g}_{\text{leak}}$, $\bar{g}_{\text{sr}}$, and $C_m$; cable $\bar{g}_{\text{leak}}$, $C_m$, and length; distal $\bar{g}_{\text{leak}}$, $\bar{g}_{\text{sr}}$, and $C_m$; and global $V_{\text{ss}}$, $k$, $m_0$, $E_{\text{sr}}$, and $E_{\text{leak}}$ were adjusted during fitting.

The data from neuron LP4 (chosen because it had the largest $R_N$ change in response to changes to $V_{\text{ss}}$, see Figure 78B) were used to fit the two models. To reduce the influence of noise in the waveform recordings, an idealized waveform trace was constructed. The average voltage response of the neuron to the current pulses (see Determining mean voltage responses to the current pulses above) was appended to itself, repeating for a total of eight cycles. The NEURON Multiple Run Fitter, with some hand-adjustment of parameters, was used to optimize model parameters until the model neuron’s voltage response to the current pulse train was nearly identical to the average response of the real LP neuron, differing by an average of 0.5 mV or less. Only the voltage response during the final pulse was considered; with a 0.2 s $\tau$, this allowed the slow conductance to reach its new steady-state mean value by the beginning of the last pulse.

The fitted models were then subjected to a full stimulus protocol of three stairstep cycles. To make each stairstep cycle visibly unique in plots, a constant 0.075 nA was added to the bias current in each subsequent stairstep cycle (Figure 81). The resulting response waveforms were processed as described above (Determining mean voltage responses to the current pulses through Statistical analyses).

Visualization

Data were plotted using gnuplot and custom software written in Perl. Final figures were prepared in Canvas (Deneba Software, Miami, FL).
Results

The response of a passive isopotential neuron to a current step of amplitude $I$ is given by an exponential charging curve,

$$V_m(t) = V_{\text{final}} + A e^{-t/\tau},$$

where $V_m$ is membrane potential, $V_{\text{final}}$ is the $V_m$ achieved after infinite time, $\tau$ is exponential time constant, and $A$ is voltage excursion maximum amplitude. $\tau = RC$ and $A = -IR$, where $R$ is neuron resistance and $C$ neuron capacitance. In such neurons $C$ can therefore be measured by fitting the charge curve with a single exponential and calculating $C = -I\tau/A$. In non-isopotential passive neurons current flows both across the membrane and among internal compartments. In such neurons the charge curve is given by a sum of exponentials

$$V_m(t) = V_{\text{final}} + \sum_{i=0}^{\infty} A_i e^{-t/\tau_i}$$

and input resistance ($R_N$) does not equal $R$, but instead $\sum_{i=0}^{\infty} R_i$, where $R_i = -A_i/I$. The time constant arising from neuron capacitance and resistance $\tau_m$ is most closely approximated (in theory, given exactly) by the slowest $\tau$ coefficient $\tau_0$ (Holmes et al., 1992; Major et al., 1993; Rall 1969) and $C$ is calculated not from $C = \tau_0/R_N$, but from $C = \tau_0/R_0 = -I\tau_0/A_0$ (Major et al., 1993).

This analysis depends on the neuron either having only passive membrane responses, or any voltage-dependent phenomena in it having dynamics faster than the passive membrane response, as otherwise the slowest term may reflect the dynamics of the slow voltage-dependent phenomena, not the passive membrane response. In the past, achieving this goal has been attempted by performing current steps from relatively negative membrane potentials (typically -50 mV in pyloric neurons), on the assumption that most depolarization-activated conductances will be inactive in this membrane potential range, and by blocking the hyperpolarization-activated current $I_h$ with cesium (e.g., Golowasch et al., 2009). However, to our
knowledge whether neuron responses under these conditions are nonetheless contaminated by voltage-dependent phenomena has not been tested.

To assess this possibility, we applied current pulses to PY and LP neurons of the lobster stomatogastric ganglion, in saline containing CsCl to block $I_h$ (Hartline and Graubard, 1992), from multiple membrane potentials ($V_{ssc}$) achieved by bias current injection. These potentials included the -50 to -60 mV range used in previous work measuring pyloric neuron capacitance (Golowasch et al., 2009). The amplitude of the voltage responses of the neurons to identical current steps changed considerably as $V_{ssc}$ was varied from -50 mV to -95 mV (Figure 73), indicating that $R_N$ changed in this voltage range (in the example shown, increasing from 1.6 to 2.2 MΩ, some 37.5%, Figure 74). A likely source of this $R_N$ change was changes in voltage-dependent currents. Because $R_N$ measurement does not measure response dynamics, this alone did not show that these currents are affecting capacitance estimation (e.g., if the currents change quickly, they would not alter $C$ estimation), but they did indicate this possibility exists. Note also that these $R_N$ changes were not due to unblocked $I_h$, since $I_h$ would decrease, not increase, $R_N$ at more hyperpolarizing membrane potentials.

PY and LP neuron membrane voltage responses to current steps were well fit by a sum of exponentials. Figure 75 shows responses to identical hyperpolarizing current steps from two $V_{ssc}$. When current steps were applied at $V_{ssc}$ more hyperpolarized than about -60 mV, the voltage responses monotonically increased (Figure 75A). Note the lack of a sag voltage during the step, demonstrating again that $I_h$ was completely blocked. The three fit line components (red, magenta, and blue) had well-separated time constants; the slowest (red) would represent neuron capacitance in the standard approach. When current steps were applied at $V_{ssc}$ more depolarized than about -60 mV, the voltage responses were no longer monotonic, but instead showed a late depolarization (Figure 75B) associated with a sign change in the amplitude of the slowest
exponential fit term (red). Because capacitance is a passive phenomenon, voltage responses must have the same sign as the charging current. Thus, the late depolarization, and the slowest exponential fit term, cannot be attributed to neuron capacitance. Because the slowest exponential term in Figure 75B is not due to capacitance, it follows that the slowest term in Figure 75A may not be either.

Another possible source of the slow responses in both cases would be a slow, depolarization-activated, voltage-dependent conductance. The slow hyperpolarization in Figure 75A is consistent with a depolarizing membrane current that slowly deactivates in response to the hyperpolarizing current step, and the slow depolarization in Figure 75B to a hyperpolarizing membrane current that slowly deactivates in response to the current step, with the switch in

![Graph](image)

*Figure 74.* Input resistance of neuron LP2 increased as $V_{ssc}$ was hyperpolarized. $R_N$ was measured by dividing the membrane voltage deflection at the end of each pulse half cycle (hyperpolarizing and relaxing phases) by pulse current. The tight clustering of points indicates that, at each $V_{ssc}$, $R_N$ was stable both within pulse trains and across the three stairstep cycles.

...
current direction being due to the $V_{ssc}$ crossing the current’s reversal potential. Consistent with this hypothesis, in the data from all steps in this neuron, $A_0$, the amplitude coefficient of the slowest exponential term (squares, Figure 76) continuously decreased as $V_{ssc}$ was depolarized from -100 mV (purple), crossing zero at $V_{ssc}$ around -50 mV (yellow), and then becoming negative at more depolarized $V_{ssc}$ (red, orange). Negative $R_0$ values cannot occur for a passive resistive element, as it implies that positive current applied to the resistor would give a negative voltage across it. Negative $R_0$ values are, however, consistent with the hypothesis of a depolarization-activated voltage-dependent conductance.

Figure 75. Membrane potential response of neuron LP2 to hyperpolarizing current steps at $V_{ssc}$ = -95 mV (A) and -35 mV (B), fit to a sum of three exponentials. Amplitude and time course of slow (red), medium (magenta), and fast (blue) terms are shown.

This decrease in $A_0$ at $V_{ssc}$ near the apparent reversal potential of -50 mV would be expected to result in $A_0$ sometimes becoming too small to fit successfully, and fits in this membrane voltage range indeed sometimes required only two terms, with three-term fits not yielding significant improvement by F test. Examination of the time constants of the three-term and two-term exponential fits showed that, as expected, in the two-term cases it was the slowest
term of the three-term fits that was missing. In the two-term fits the two terms were therefore placed in the middle and fastest term classes. As there was no overlap between the \( \tau_0 \) (approx. 0.2 s), \( \tau_1 \) (approx. 0.02 s), and \( \tau_2 \) (approx. 0.002 s) classes, this classification was unambiguous.

Figure 76. \( \tau \) and \( A \) coefficients from neuron LP2’s fitted exponential terms, colored by \( V_{ssc} \) (color scale bar, right)

Slow, medium, and fast exponential terms are represented by squares, circles, and triangles, respectively. Isocapacitance curves (curved black lines) for each temporal class were generated from \( \tau = AC/I_{pulse} \), where \( C \) is the mean of the capacitive coefficients within each class (see Materials and Methods). The failure of the slow term class data (\( \tau_0 \) and \( A_0 \)) to lie on their corresponding isocapacitance line, and \( A_0 \) becoming negative at \( V_{ssc} \) around -50 mV, suggests the presence of a voltage-dependent process.

During the 60 s equilibrating time after setting a new \( V_{ssc} \), the hypothesized slow conductance would open or close and assume a new steady state activation value by the time the current pulses began. \( A_0 \) would thus reflect only the change in the voltage-dependent current in
response to the roughly 10 mV membrane potential changes occurring during the current pulses themselves. With the hyperpolarizing current step at the beginning of the pulse, the conductance would close slightly, causing a hyperpolarization at $V_{ssc}$ more hyperpolarized than the conductance’s reversal potential and a depolarization at $V_{ssc}$ more depolarized than the conductance’s reversal potential. With the depolarizing step back to $V_{ssc}$, the conductance would open slightly, yielding the opposite effect. Importantly, since the hypothesis is that the current opens and closes more slowly than the capacitive time constant of the neuron, the slowest exponential term ($A_0$ and $\tau_0$) would thus not represent neuron capacitance.

The changes in the amount of the conductance that was open during the 60 s equilibrating phase would change the neuron’s resistance, increasing it at more hyperpolarized $V_{ssc}$. In addition to making $A_0$ and $\tau_0$ voltage dependent as explained above, this change in neuron resistance would also be expected to change the $A_1$ and $\tau_1$ coefficients (which under this hypothesis would represent true neuron capacitance) because of their dependence on neuron resistance. $A_1$ and $\tau_1$ indeed both decreased as $V_{ssc}$ varied from -95 mV (purple) to -30 mV (red), $A_1$ from around 3.5 mV to around 1 mV and $\tau_1$ from around 0.02 s to around 0.006 s (circles, Figure 76). Changes in neuron resistance would also likely change the $A_2$ and $\tau_2$ coefficients which represent the neuron settling response as current spreads throughout the neuron’s dendritic tree, although predicting the effect that this would have on $A_2$ and $\tau_2$ is difficult (Major et al., 1993).

A slow voltage-dependent conductance would therefore be predicted not only to introduce a non-capacitive slowest $A_0/\tau_0$ term in the multi-exponential fits, but also to cause voltage-dependent changes in the $A_1/\tau_1$ term from which neuron resistance and capacitance should be calculated, and the $A_2/\tau_2$ term that represents current spread throughout the dendritic tree. Importantly, however, true neuron capacitance should be constant as membrane potential changes. Since at all $V_{ssc}$ the same current pulse amplitude ($I$) was injected, it follows from the
relationship $C_i = -I/\tau_i$ and hence $\tau_i = -C_i A_i/I_i$, that, for a constant $C_i$, $\tau$ and $A$ should be linearly related. We tested this prediction for each $A_i/\tau_i$ class by calculating the mean of each term’s $C_i$ coefficients across all pulse trains at all $V_{sc}$ and using this mean to plot isocapacitance lines for each mean $C_i$ value (black lines in Figure 76). The data points for the $A_1$ and $\tau_1$ and $A_2$ and $\tau_2$ terms closely followed linear relationships (lines are curved because plot is semi-logarithmic). The $A_0$ and $\tau_0$ data points, alternatively, did not, further supporting the hypothesis that the $A_0/\tau_0$ term represents a non-capacitive phenomenon.

Using $\tau_i$ and $A_i$ to calculate each term’s resistive and capacitive coefficients gave results consistent with the data in Figure 76 and the hypothesis presented above. With respect to resistance, $R_0$ (squares, Figure 77A) decreased and then became negative as $V_{sc}$ was depolarized, crossing a reversal potential at about -50 mV. $R_1$ (circles) and $R_2$ (triangles) varied linearly with $V_{sc}$ but always remained positive. At very depolarized $V_{sc}$, where $A_0$ becomes negative, calculated $C_0$ becomes negative, impossible for a true capacitance. We therefore plotted $C_i$ values only for $V_{sc}$ more hyperpolarized than -45 mV. Because of the very large differences between the $C_i$ coefficients, we present these data in both linear (Figure 77B) and semi-logarithmic (Figure 77C) plots. $C_0$ (squares) depended strongly on $V_{sc}$, varying from approximately 1,500 nF at -95 mV to 10,500 nF at -47.5 mV (some 6-fold). $C_1$ (circles) and $C_2$ (triangles) varied much less, each changing by approximately 10% of their mean values.

The data in the previous figures are from a single LP neuron. In experiments on three other LP neurons and five PY neurons, the slowest fit terms were again not due to neuron passive membrane properties, but instead to a phenomenon consistent with slow voltage-dependent conductances. All neurons showed voltage-dependent variations in $R_N$ (Figure 78; divided into two panels due to the large $R_N$ variation across the neurons). Importantly, in almost all cases $R_N$ changed with hyperpolarization in the -50 to -60 mV range at which capacitance measurements
are typically made. These $R_N$ changes are consistent with residual voltage-dependent conductances active in this voltage range (see Discussion), which could interfere with capacitance measurements.

Figure 77. Resistive coefficients ($A$) shown in a linear plot, and capacitive coefficients shown in linear ($B$) and semilog ($C$) plots, derived from the $\tau$ and $A$ coefficients of neuron LP2. Coefficients were calculated as $R_i = A_i / I_{\text{pulse}}$ and $C_i = \tau_i / R_i$. Slow, medium, and fast exponential terms are represented by squares, circles, and triangles. Fits to capacitive ($C = m \cdot V_{ssc} + b$) and resistive ($R = m \cdot V_{ssc} + b$) coefficients are shown (lines). Linear fits appear curved in panel $C$ because plot is semi-logarithimic. Triangles in panel B are not visible beneath circles.
Fits to the charging curves of these neurons again showed three $\tau$ classes, one around 0.2 sec, a second around 0.02 s, and a third around 0.002 s, and $A_0$ values that decreased with depolarization and eventually became negative (data not shown). The resulting $R_0$ sign changes occurred between -42.6 mV and -61.5 mV (-51.4±5.85 mV, mean ± SD), consistent with a conductance reversing at these potentials (Figure 79, grouped again by neuron $R_N$ as in Figure 78). $C_0$ again showed large voltage dependence in all neurons (Figure 80). These variations were significant for all neurons and the slopes of the fits differed from zero (Table 12). $C_1$ and $C_2$ showed much smaller changes, both in absolute variation and relative to the mean values of the coefficients. The $C_1$ variations were significant for all neurons and the slopes of the fits differed from zero. Four of the nine neurons did not show a significant dependence of $C_2$ on $V_{ssc}$, and the slopes of the significant $C_2$ fits did not differ from zero.

**Figure 78.** Input resistance of all neurons at different $V_{ssc}$ values, separated for clarity into low ($A$) and high $R_N$ ($B$) groups.

Error bars show standard deviation of each pulse train’s mean $R_N$ and $V_{ssc}$. All LP neurons showed increased input resistance at hyperpolarized potentials, as did two PY neurons (PY4 and PY5) during the first stairstep cycle (descending magenta and green traces, $B$).
Figure 79. $R_0$ vs. $V_{sec}$ for all neurons, separated into low (A) and high-$R_N$ (B) groups

Significant linear fits were found for all neurons. $R_0$ sign changed around -50 mV in all neurons, suggesting a reversal potential

$R_0$ crossing zero (Figure 79) and the large variation in $C_0$ (Figure 80A) are inconsistent with this term of the exponential fits representing true $R$ and $C$. The $R_1/C_1$ coefficients, as the next slowest component, more likely represent these parameters. However, $C_1$ nonetheless shows a slight voltage dependence, which might not be expected for true $R/C$ values. To investigate this issue under completely controlled conditions, we constructed ball and stick (non-isopotential) and single compartment (isopotential) models in NEURON. Fitted parameters are reported in Table 13. These models included a slow, depolarization-activated conductance with a reversal potential near -50 mV, consistent with the experimental data. The models reproduced all salient features of the experimental data. In particular, their $R_N$ increased with hyperpolarization (Figure 81A, data from ball-and stick model). In the ball and stick model, fits to the voltage responses produced three exponential terms with $\tau$ values near 0.2, 0.02, and 0.001 s. The slowest term did not follow an isocapacitance curve, but the second slowest did (the third having too little variation to assess) (Figure 81B). In both the ball and stick (Figure 81C) and isopotential (Figure 81D)
models, the second-slowest capacitive coefficient ($C_1$) showed voltage dependence with a slight negative slope, consistent with the experimental data. As $C$ is known in these models (ball and stick, 9.4 nF; isopotential, 7.8 nF), it is clear that the second-slowest term is the correct term to calculate neuron capacitance. Thus, these data show that in neurons with contaminating slow voltage dependent conductance(s), these conductance(s) add slight voltage dependence to the coefficient representing true neuron capacitance. Notably, $C_1$ equals $C$ near the reversal potential of the slow conductance. This suggests that, in neurons with such conductances, capacitance measurement should be made at the conductance’s reversal potential.

Figure 80. $C_0$ (A), $C_1$ (B), and $C_2$ (C) vs. $V_{ssc}$ for all neurons.

Linear fits are shown where significant ($p < 0.05$). Because $R_0$ became negative at $V_{ssc}$ more depolarized than -50 mV, the $C_0$ terms at these depolarized $V_{ssc}$ values are not shown, and were not used in the fits.
Based on these model observations, we calculated \( C \) in real neurons (Table 14) in two ways. For neurons in which \( C_1 \) significantly varied with membrane potential, we took as membrane capacitance the values of the \( C_1 \) coefficient linear fits (Figure 80B) at the membrane potential at which \( R_0 \) crossed zero (\( R_0 \)’s reversal potential, Figure 79) (Table 14). For neurons in which \( C_1 \) did not significantly vary with membrane potential, we used the mean of the \( C_1 \) values measured at membrane potentials closest to the \( R_0 \) zero-crossing. Mean PY neuron \( C \) was 5.4±1.7 nF (area 0.0060±0.0019 cm\(^2\)) and mean LP neuron \( C \) was 12±3.6 nF (area 0.013±0.0040 cm\(^2\)), in both cases assuming a specific capacitance of 0.9 µF/cm\(^2\) (Gentet et al., 2000). PY and LP neuron mean \( C \), and thus mean areas, differed at \( p < 0.05 \) (Student’s \( t \)-test with unequal variance assumed), consistent with optical measurements showing that PY neurons are smaller than LP neurons (Thuma et al., 2009).

Possible alternative explanations

The slow voltage-dependent phenomenon observed here could be an artifact of the fitting procedure. We tested this possibility by generating artificial data analogous to the curves shown in Figure 75 including noise but without a slow component. The fitting procedures found no slow component in these data. The phenomenon could also arise from electrical artifacts arising from the electrometer, reference electrode, or microelectrodes. We tested this possibility by repeating the experiments using an electronic model cell connected, via agar bridges, to the reference and microelectrodes. Fits to these data again found no slow voltage-dependent terms. The data reported here are thus not due to fitting or experimental capacitive artifacts.

The slow component could also arise from a slow continuous drift in membrane potential. Within each individual pulse, such a drift would add to the membrane potential change induced by the current pulse. If large enough, the fitting procedure would fit this slow change with an exponential term. If this were the case, since the direction of the drift would be the same in the
hyperpolarizing and relaxing phases of the step, the sign of the slow term amplitude coefficient ($A_0$), and thus $R_0$, would reverse between these phases. Except when the voltage changes induced by the current steps straddled the slow component’s reversal potential (e.g., the blue squares at $V_{ssc} = -55$ mV), the $R_0$ values of the fits to these two phases instead always had the same sign (Figure 79). The slow component is therefore not due to a slow continuous membrane potential drift.
Figure 81. Response of optimized NEURON models to stairstep protocols

Ball-and-stick model input resistance ($A$), $\tau$ and $A$ coefficients ($B$), capacitive coefficients ($C$), and single-compartment model capacitive coefficients ($D$) are shown. In addition to standard passive properties, both models included a non-inactivating voltage-dependent conductance designed to behave similarly to the active component observed in our experimental data. The $C_1$ coefficients of both models showed slight voltage dependence with a negative slope, consistent with experimental data. Ball-and-stick models are morphologically simple compared to real pyloric neurons. Thus, the fast exponential term, reflecting current flow due to non-isopotentiality, varies only slightly with $V_{ssc}$, with changes to $\tau_2$ and $A_2$ coefficients not being visible (overlapping triangles, panel B) and the $C_2$ coefficient varying only 0.0017 nF/mV (C). The $C_2$ coefficient is absent in the isopotential model fits (panel D) because there is no such current flow.
Table 12
Capacitive Coefficients for All Neurons

<table>
<thead>
<tr>
<th>Neuron</th>
<th>$C_0$ slope</th>
<th>%/mV</th>
<th>$C_1$ slope</th>
<th>%/mV</th>
<th>$C_2$ slope</th>
<th>%/mV</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nF/mV</td>
<td></td>
<td>nF/mV</td>
<td></td>
<td>nF/mV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LP1</td>
<td>129</td>
<td>2.4</td>
<td>-0.0816</td>
<td>-0.52</td>
<td>0.612**</td>
<td>-0.00226</td>
<td>-0.13</td>
</tr>
<tr>
<td>LP2</td>
<td>223</td>
<td>4.3</td>
<td>-0.064</td>
<td>-0.41</td>
<td>0.651**</td>
<td>0.00837</td>
<td>0.37</td>
</tr>
<tr>
<td>LP3</td>
<td>200</td>
<td>5</td>
<td>-0.0658</td>
<td>-0.53</td>
<td>0.630**</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>LP4</td>
<td>47.4</td>
<td>7.8</td>
<td>-0.0242</td>
<td>-0.33</td>
<td>0.607**</td>
<td>-0.00435</td>
<td>-0.24</td>
</tr>
<tr>
<td>PY1</td>
<td>84.6</td>
<td>2.8</td>
<td>0.620*</td>
<td>NS</td>
<td></td>
<td>0.0143</td>
<td>0.45</td>
</tr>
<tr>
<td>PY2</td>
<td>27.9</td>
<td>2</td>
<td>0.499*</td>
<td>-0.0717</td>
<td>-0.94</td>
<td>0.778**</td>
<td>0.00362</td>
</tr>
<tr>
<td>PY3</td>
<td>76.1</td>
<td>4.2</td>
<td>0.548**</td>
<td>-0.096</td>
<td>-1.1</td>
<td>0.452**</td>
<td>NS</td>
</tr>
<tr>
<td>PY4</td>
<td>39.5</td>
<td>2.9</td>
<td>0.540**</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>PY5</td>
<td>30.8</td>
<td>6</td>
<td>0.600*</td>
<td>-0.0233</td>
<td>-0.66</td>
<td>0.846**</td>
<td>NS</td>
</tr>
</tbody>
</table>

For each fitted exponential term, a linear fit ($C = m \times V_{ssc} + b$) was made to the voltage dependence of the capacitive coefficients (intercepts not shown), with slope additionally expressed as change relative to the mean of the capacitive coefficients (%/mV). R = correlation coefficient. *: $p<0.05$  **: $p<0.01$; NS: not significant
Table 13

NEURON Model Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Isopotential</th>
<th>Ball and Stick</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{sr}$</td>
<td>-58 mV</td>
<td>-60 mV</td>
</tr>
<tr>
<td>$V_{1/2}$</td>
<td>-43 mV</td>
<td>-12.3 mV</td>
</tr>
<tr>
<td>$k$</td>
<td>16.8 mV</td>
<td>22.1 mV</td>
</tr>
<tr>
<td>$m_0$</td>
<td>0.09 (unitless)</td>
<td>0.03 (unitless)</td>
</tr>
<tr>
<td>$E_{leak}$</td>
<td>-68 mV</td>
<td>-68 mV</td>
</tr>
<tr>
<td>Soma $g_{sr}$</td>
<td>0.24 mS/cm$^2$</td>
<td>0.63 mS/cm$^2$</td>
</tr>
<tr>
<td>Soma $g_{leak}$</td>
<td>0.04 mS/cm$^2$</td>
<td>0.04 mS/cm$^2$</td>
</tr>
<tr>
<td>Soma $c_m$</td>
<td>5 $\mu$F/cm$^2$</td>
<td>15.25 $\mu$F/cm$^2$</td>
</tr>
</tbody>
</table>

Soma dimensions 50 $\mu$m long, 1000 $\mu$m dia. 50 $\mu$m long, 50 $\mu$m dia.

Cable $g_{leak}$ 0.5 mS/cm$^2$

Cable $C_m$ 5 $\mu$F/cm$^2$

Cable dimensions 200 $\mu$m long, 10 $\mu$m dia.

Distal $g_{sr}$ 0.8 mS/cm$^2$

Distal $g_{leak}$ 0.015 mS/cm$^2$

Distal $C_m$ 5 $\mu$F/cm$^2$

Distal dimensions 50 $\mu$m long, 1000 $\mu$m dia.

Axial resistance ($R_a$) = 35.4 $\Omega$ cm in both models
Table 14

$R_0$ Zero Crossing, Capacitance, and Area Estimates for All Neurons

<table>
<thead>
<tr>
<th></th>
<th>$R_0$=0 mV</th>
<th>C nF</th>
<th>Area cm$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP1</td>
<td>-49.1</td>
<td>14.6</td>
<td>0.0163</td>
</tr>
<tr>
<td>LP2</td>
<td>-53.3</td>
<td>14.8</td>
<td>0.0164</td>
</tr>
<tr>
<td>LP3</td>
<td>-52.2</td>
<td>11</td>
<td>0.0127</td>
</tr>
<tr>
<td>LP4</td>
<td>-52.0</td>
<td>7.1</td>
<td>0.00788</td>
</tr>
<tr>
<td>PY1</td>
<td>-43.4</td>
<td>3.87</td>
<td>0.0043</td>
</tr>
<tr>
<td>PY2</td>
<td>-55.6</td>
<td>7.26</td>
<td>0.00806</td>
</tr>
<tr>
<td>PY3</td>
<td>-42.6</td>
<td>6.72</td>
<td>0.00746</td>
</tr>
<tr>
<td>PY4</td>
<td>-53.1</td>
<td>5.93</td>
<td>0.00659</td>
</tr>
<tr>
<td>PY5</td>
<td>-61.5</td>
<td>3.44</td>
<td>0.00382</td>
</tr>
<tr>
<td>All LP</td>
<td>-51.6</td>
<td>11.9±3.6</td>
<td>0.0132</td>
</tr>
<tr>
<td>All PY</td>
<td>-51.2</td>
<td>5.4±1.7</td>
<td>0.006</td>
</tr>
<tr>
<td>All</td>
<td>-51.4</td>
<td>8.3±4.2</td>
<td>0.0092</td>
</tr>
</tbody>
</table>

The $V_{ssc}$ at which $R_0$ crossed 0 ($R_0=0$) was calculated from linear fits to the $R_0$ coefficients. Where $C_1$ linear fits were significant (p < 0.05), neuron capacitance ($C$) estimates were taken from the value of the fitted line at this $R_0=0$ potential; otherwise, the mean of all $C_1$ values at $V_{ssc}$ closest to the $R_0=0$ potential were used. Area estimates were made assuming a specific capacitance of 0.9 µF/cm$^2$ (Gentet et al., 2000).
Discussion

We have shown that pyloric neurons contain a slow, voltage-dependent phenomenon that complicates current-clamp based measurement of neuron capacitance by appearing as the slowest term in multi-exponential fits. This phenomenon had an apparent reversal potential and was associated with changes in neuron input resistance, features consistent with voltage-dependent conductances. The second-slowest term, presumably representing true neuron capacitance, was also slightly voltage dependent. Computational modeling demonstrated that the true capacitance term of model neurons possessing a slow voltage-dependent conductance showed similar voltage dependence. These data indicate that in pyloric neurons the second-slowest term represents neuron capacitance. In general, the capacitive term will not necessarily be the second-slowest. The characteristic that identifies the capacitive term is instead that it is the slowest term without appreciable voltage dependence.

Past current-clamp based measurements of neuron capacitance in this and other systems (Arsenault et al., 2011; Firestein and Werblin, 1987; Golowasch et al., 2009; Liao and Lee, 2011; Roth and Häusser, 2001; Szabo et al., 2010) were made at single membrane potentials on the assumption that no voltage-dependent processes were active at these potentials (i.e., without checking for voltage dependence), and always used the slowest term of the exponential fits, even in cases in which, as here, good fits to a single neuron’s membrane voltage responses sometimes required two, and other times three, terms. Our data show this treatment is incorrect for pyloric neurons, and suggest that capacitance measurements in other systems could be similarly compromised. Given the importance of capacitance in neuron activity (Figure 72), such errors in capacitance may be a major cause of the failure of many ‘data-based’ models to reproduce the activities of the very neurons from which the model parameters were obtained.
**Comparison to standard technique**

It is illuminating to consider what happens when the standard current-clamp capacitance measurement technique is applied to our data. Measurements are typically performed at a membrane potential of -50 mV. Because this is near the reversal potential of the slow, voltage-dependent phenomenon described here, at this potential the contribution of the phenomenon is so small that often only two-term fits are obtained. The capacitive coefficient calculated from the slowest term in these two-term fits therefore correctly represents neuron capacitance. When three-term fits are required, however, the capacitive coefficient of the slowest term reflects the dynamics of the slow voltage-dependent phenomenon. Averaging the $C_0$ coefficients from the two and three term fits would therefore yield an erroneously high capacitance estimate with a large coefficient of variance.

Applying the standard measurement technique to the data across all our neurons, but only from the pulse trains at $V_{ssc} = -50$ mV, confirmed this. Among LP neurons, significant three-term fits were obtained in 17 of 22 measurements, with the remaining being two-term fits. Among PY neurons, significant three-term fits were obtained in 19 of 24 measurements. Calculating capacitance using the slowest terms from both two- and three-term fits gave a mean capacitance of 6697±5504 (standard deviation) nF in LP neurons, and 1954±1962 nF in PY neurons. These capacitance estimates differ from those made with our technique in two ways. First, they are some 550-fold (LP) and 350-fold (PY) larger than our estimates of 11.9±3.6 nF (LP) and 5.4±1.7 nF (PY) (*Table 14*). Second, their coefficients of variation are larger, being 0.8 (LP) and 1.0 (PY), compared to ours of 0.3 (both LP and PY). This large coefficient of variation arises from the slowest terms of the two and three term exponential fits being treated as equivalent in the
standard method. Binning confirmed that two distinct capacitive coefficient classes exist (Figure 82). As expected, all low capacitance measurements came from two-term fits and all high capacitance measurements from three-term fits.

![Figure 82. Bimodal distribution of “naïve” neuron capacitance estimates](image)

Data from $V_{ssc} = -50$ mV (typical in capacitance measurements). Charge curves were fit to 2 or 3 term exponential fits as needed (see Materials and Methods). The slowest fit term was used regardless of its dynamic class. $x$’s represent capacitances of each fit. Histogram with 16 bins. Two populations are present. The smaller was from the two-term fits, in which the slow, voltage-dependent phenomenon amplitude was too small for the fitting procedure to find. The larger population was from the three-term fit.

**Comparison to prior pyloric work**

The good fits to the voltage responses (Figure 75) and relative isocapacitance of the middle and fastest terms (Figure 76; Figure 77 B,C; Figure 80 B,C), agree with the primary conclusion of Golowash et al. (2009) that current-clamp techniques measure neuron capacitance well. However, our data indicate that proper use of this technique requires performing it at multiple membrane potentials in order to identify which exponential term represents $R$ and $C$. 
Failure to do so can lead to hundred-fold overestimates of capacitance and large coefficients of variation. Prior current-clamp estimates of pyloric neuron capacitance using the standard technique reported capacitance measurements hundreds of times larger than those expected from anatomical data, with coefficients of variation between 0.6 and 1 (Golowasch et al., 2009). A retrospective analysis examining these data for multi-modal capacitance distributions (Figure 82) would help resolve whether they are compromised. However, as individual data points were not provided in this work, we were unable to perform this analysis.

Even with correct term identification, our estimates of pyloric neuron surface area of 0.006 (PY) to 0.01 (LP) cm$^2$ are still two to three times larger than the largest surface areas of dye-filled lobster pyloric neurons (0.003 cm$^2$, data from our lab, see (Thuma et al., 2009). However, dye fills are likely to be incomplete, particularly for small fibers, and stomatogastric neurons possess processes whose diameter is below optical resolution (King, 1976). Given these difficulties, this discrepancy is not unreasonable.

**Nature of slow voltage-dependent phenomenon**

We have treated this phenomenon as if it were a non-inactivating, depolarization-activated, slowly opening conductance. Given $R_0$’s reversal and the voltage-dependent $R_N$ changes, this treatment is not unreasonable. However, these data are not evidence that the slow phenomenon is a single conductance, and we are unaware of any conductance with the properties found in the NEURON model optimization (Table 13). An alternative explanation arises from the fact that the activation curves of multiple stomatogastric neuron conductances (persistent sodium, two calcium, the delayed rectifier, the calcium-activated potassium, and the A conductance) all begin to open at membrane potentials more hyperpolarized than -75 mV (Elson and Selverston, 1997; Prinz et al., 2003; Turrigiano et al., 1995). In the voltage ranges used here these conductances show little inactivation, and their kinetics of activation and inactivation are slow.
The steady-state values of these conductances over the voltages used here are small, but for calcium and sodium currents the driving forces are large, and the fitting process reliably identifies small amplitude terms. Additionally, many neurons possess ion channels with very slow inactivation kinetics, e.g., the dendrotoxin-sensitive Kv1.2 potassium channel (Shen W et al., 2004). Similar channels could contribute to the identified ionic conductances in pyloric neurons. The slow voltage dependent phenomenon we have identified here thus likely arises from the sum of the small, but not zero, currents flowing through these conductances even at the hyperpolarized membrane voltages used here. It is also important to stress that the voltage dependencies of stomatogastric neuron conductances are not different from those typically observed in other neurons. These considerations thus suggest that the difficulties in measuring neuron capacitance that we have identified here likely apply across preparations.

The one conductance the slowest term cannot arise from is unblocked $I_h$, as, in the range of $V_{ssc}$ used here, $I_h$ would induce a depolarizing sag at hyperpolarized $V_{ssc}$ and a hyperpolarizing component at depolarized $V_{ssc}$. The actual effect of the slow component was precisely opposite (Figure 75).

Remaining limitations in current-clamp based method of capacitance estimation

We have identified a major potential problem in the most appropriate capacitive measurement technique for most neurons (Golowasch et al., 2009) and provided a solution: sort the time constants of the multiple exponential fits into classes, identify which classes are voltage independent, and calculate neuron capacitance from the slowest voltage-independent class. Although this will generally work well, there remains one case that, to our knowledge, cannot be overcome electrically. If a voltage-dependent phenomenon exists, but its time constant is very similar to the time constant of the true capacitive term across the $V_{ssc}$ range (e.g., if the upper left data points in Figure 76 had $\tau$ values around 0.01 instead of 0.5), then the fitting algorithm will
not separate the two terms. Instead, it will fit the sum of these two changes with a single exponential term. In Figure 76, this would result in the $A_1$ red and orange points being shifted left and the purple, blue, and green points right.

In pyloric neurons, the amplitude of the voltage dependent phenomenon is so small that such shifts would change the $A_1/\tau_1$ curve, and the voltage dependence of the $R_1$ points in Figure 77C, only slightly (the $R_0$ points would disappear, having been subsumed into the sum described above). If $A_0$ were large, however, the $R_1$ points in Figure 77C would show a large voltage dependence and hence be rejected. The slowest of the settling time constants would therefore be identified as representing neuron capacitance. This would substantially underestimate capacitance, giving a neuron capacitance of approximately 2 nF, some 5 fold lower than our estimate of approximately 10 nF (Table 14). However, using the slowest, but voltage-dependent, term, in which the $A$ would reflect both capacitor charging and the voltage-dependent process, would also lead to substantial capacitance estimate errors. Thus, in the unlikely event that a neuron possesses a large amplitude (relative to the voltage response due to neuron capacitance) voltage-dependent phenomenon whose time constant is close to the neuron charging time constant, neither our, nor any similar capacitance measurement technique, will yield accurate results.

*Implications for correct capacitance estimations and summary*

In choosing among competing methods for capacitance measurement, two sources of error must be considered. The first is current flow among dendritic processes in non-isopotential neurons. The second is the presence of slow, voltage-dependent phenomena, as identified here. Generally speaking, methods that separate different dynamic components of the neuron response are more robust to non-isopotentiality at the expense of susceptibility to error from residual voltage-dependent phenomena. Voltage-clamp methods using step or ramp voltage changes are
less accurate than current-clamp methods for non-isopotential neurons (Golowasch et al., 2009). However, because they rely on the total amplitude of the clamp currents, the error introduced by slow, voltage-dependent current should scale approximately with the current’s amplitude (Taylor, 2011). Voltage-clamp methods based upon sinusoidal driving (Gillis, 1995) are similarly susceptible to non-isopotentiality errors, but again robust to slow, voltage-dependent currents provided they are small in amplitude. Where accurate neuron morphology is known a final method is optimizing multi-compartmental neuron model membrane parameters with experimental data (Roth and Häusser, 2001). This technique, though computationally demanding, will work well provided the morphological description is accurate and the membrane is passive, but introduce error that scales with slow, voltage-dependent current amplitude.

Given the difficulties in obtaining accurate neuron morphologies, and the fact that most neurons are non-isopotential, these considerations agree with Golowasch et al. (2009) that current-clamp methods are optimal in most cases. However, these methods can give highly erroneous capacitive estimates when slow voltage-dependent phenomenon are present. Because even very small amplitude processes can be reliably identified with multi-exponential fits, these concerns apply even when slow phenomena contribute little to total membrane response. Correct current-clamp based estimation of neuron capacitance should therefore be performed at multiple membrane potentials to look for changes in input resistance, voltage dependence of putative capacitance parameters, and evidence of reversal of the associated amplitude coefficient. The slowest term without these phenomena, measured at potentials where their contaminating influence is least (e.g., the reversal potential of slow voltage dependent phenomenon) should be used to estimate capacitance.
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Disclosures

The authors have no conflicts of interest.
CHAPTER 6: DETAILED ANATOMICAL MODELS OF PYLORIC NEURONS OBTAINED THROUGH SEMI-AUTOMATIC TRACING ARE NOT ISOPOTENTIAL UNDER PERTURBATION

Author Contributions

This research was an offshoot of anatomical research into pyloric neuron morphology. K.H.H. developed the suite of software for semi-automatic tracing of confocal neuron images using public domain segmentation and visualization software. W.E.W. developed the statistical models for evaluating appropriate contour levels used to obtain dendrite diameters. J.B.T. performed all dissections, dye fills and confocal microscopy. W.E.W. performed all traces of neurons using this software suite, and designed and performed the experiments assessing isopotentiality using the NEURON models. S.L.H. contributed to subsequent research design and interpreted experimental results. W.E.W. prepared initial versions of figures, S.L.H. prepared final versions. W.E.W. drafted manuscript; W.E.W. and S.L.H. edited and revised manuscript and approved final version.

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Introduction

In prior chapters, I showed that single-compartment pyloric model neurons can produce similar activity despite having different parameters (*Chapter 2*), and thus, attempting to discover the conductance parameters of a neuron by analysis of its free-running activity may not be possible. With perturbation, however, model neurons with different parameters typically produce different responses. Using an evolution strategy, perturbations were empirically designed to distinguish neurons on the basis of their conductance complement. Testing with a single compartment neuron model showed that these perturbations were effective at distinguishing neurons on the basis of their voltage-dependent conductance complement (*Chapter 3*). I then showed that the empirically optimized voltage clamp perturbation could elicit sufficient information from real neurons to classify them based on their perturbed activity. This classification recapitulated the biologically-determined neuron classification, with PD, VD, and IC neurons behaving in characteristic ways to different parts of the perturbation (*Chapter 4*).

A major goal of computational modeling is measuring the conductance parameters by subjecting a single real neuron to a complicated perturbation, recording its response, fitting a neuron model to the same stimulus and response, and then reading the parameters of the model. Model fitting is typically attempted using a heuristic method such as an evolution strategy to navigate the parameter space and find the appropriate regions where the model’s behavior matches the real neuron. This has not yet been achieved in pyloric neurons. Initial experiments using white noise perturbations (Hobbs and Hooper, 2008) failed to elicit sufficient voltage-dependent currents to permit parameter measurement. When different perturbations were evaluated for their utility in pathfinding, i.e., facilitating parameter discovery, (*Chapter 3*) I showed that white noise is an especially poor voltage clamp perturbation. The optimized
perturbations, however, demonstrated good performance for facilitating pathfinding in a single
compartment neuron model.

Good performance in a single compartment model does not necessarily imply good
performance in real neurons, because such models are gross simplifications of the morphological
complexity of real neurons. Detailed anatomical models made from real neurons show they can
contain hundreds or thousands of compartments. Even when such complexity is represented in
neuron models, simplifying assumptions are typically made about membrane conductances, e.g.,
assuming that the $g_{\text{max}}$ parameters of the various conductances are identical across compartments,
at least throughout large sections of the neuron. More generally, the morphological complexity is
reduced to a few electrical compartments, or even one. Reducing complexity to a few or single
compartments is appropriate if the region of the neuron being modeled as a single compartment is
relatively isopotential, i.e., having very similar membrane potential throughout the region. In
isopotential regions, the voltage-dependent conductances sense and respond to similar membrane
potentials. With non-isopotential regions, where voltage-dependent conductances in proximal
and distal locales do not sense the same membrane potential, and where current flow among
regions is appreciable, modeling the region as a single electrical compartment introduces errors.
If for example the membrane potential at the proximal end of a region is sufficient to activate a
voltage-dependent conductance but the potential at the distal end is not, and yet that region is
modeled as if it is entirely at the proximal potential, then the model will overestimate the
amplitude of the voltage-dependent conductance. It is thus critical when making models with an
anatomical basis to consider whether the model has a sufficient number of compartments to
prevent these errors.

Prior work in pyloric neurons of decapod crustaceans (Chapter 5) has established that
these neurons respond to current-clamp steps with a charge curve which is appropriately fit as the
sum of multiple exponentials. As passive, isopotential neurons respond to current steps with a simple exponential charge curve (Rall et al., 1992), this implies that pyloric neurons are not isopotential, with the faster exponential terms occurring due to current flow within the neuron. However, these measurements in pyloric neurons in *P. interruptus* are complicated by the presence of a residual active component, *(Chapter 5)*, making it difficult to draw inferences about isopotentiality under various conditions from electrophysiological data alone.

An alternative to inferences based upon electrical capacitance measurement is constructing passive models from anatomical data which faithfully reproduce the full morphological complexity of the neuron. This is typically done by staining the neuron with an appropriate dye, visualizing it under a microscope, and tracing it in three dimensions to create a model using hardware and software such as Neurolucida (Glaser and Glaser, 1990). Assuming that the dendritic arbor is sufficiently visualized and traced, these models can then be used to estimate the membrane potential throughout the neuron in response to stimulation. Where most of the membrane surface area remains within a narrow voltage range, a single electrical compartment can be utilized with little introduction of error.

One complication in this procedure is the inability to resolve and trace fine neural components due to inadequate dye penetration or resolution limitations. If the capacitance of the neuron type, and thus its membrane surface area, is known, it is possible to estimate the area of missing membrane. The anatomical model can then be corrected by extrapolating it to add missing processes, or by changing the capacitance and conductance of the membrane to simulate additional surface area. In the absence of evidence of the location of the missing membrane area, the choice in how to make these adjustments is somewhat arbitrary, thus multiple methods should be employed to establish what effect these choices have upon estimates of isopotentiality.
The fidelity of these models relies upon accurate tracing and digitization of neural structures. Traditional tracing systems rely strongly upon operator experience and attention to detail. Results differ significantly depending on which system is used, due to operator experience and fatigue, differences in ability to visualize the data, and similar factors (Anderson et al., 2010; Holmes et al., 2006). A separate goal of this research was thus to develop automatic tracing software to facilitate accurate, repeatable digitization of neuronal morphology, and reduce the variability in results due to operator choices. Considerable sophistication in image processing and segmentation algorithms is available in open source libraries to support such an endeavor. This software could be used to reconstruct anatomical models from confocal stacks or equivalent source data in a semi- or fully-automated fashion.

Accurate anatomical models of pyloric neurons would thus permit investigation of the question of whether these neurons are isopotential in experimental conditions. During voltage clamp, input resistance changes substantially with membrane potential, presumably due to activation of voltage-dependent conductances. If pyloric neurons are not isopotential during more depolarized voltage clamping, it is likely that the conductance-optimized perturbations will not effectively constrain membrane potential throughout the dendritic arbor. This would mean that portions of the neuron would react more similarly to current clamp than voltage clamp, with intrinsic activity and state contributing to the neuron’s voltage response. The optimized perturbations did not demonstrate good results in current clamp conditions (Chapter 3), and thus it is important to resolve the question of whether pyloric neurons are isopotential under voltage clamp conditions.

Overview of research

The question of pyloric neuron isopotentiality was investigated in several steps. A semiautomatic neuron tracing program was developed in C using open source visualization and
segmentation libraries. The program was used to trace the center lines of the dendritic arbors in confocal image stacks of dye filled pyloric neurons. The software used these “skeleton” traces to automatically build highly detailed morphological reconstructions of the original neurons. These reconstructions were then converted into multi-compartment electrical models containing only passive properties, i.e., membrane capacitance and conductance, and axial resistance.

Anatomical models of this nature frequently underestimate the total membrane surface area. Dye penetration is often incomplete, especially in fine terminal processes, and very small features cannot be adequately visualized or recorded using confocal microscopy. If the anatomical models do not adequately represent the real neuron morphology, inferences drawn from them are unlikely to be correct. Thus, the anatomical models were extended in one of two ways to compensate for missing portions of the neuron. One method was to adjust the capacitance and conductance in the different compartments of the model in various ways to simulate the missing membrane area. The second was to automatically add compartments to the model, extending the neuron’s branching pattern.

These adjusted passive models of pyloric neurons were then used to investigate whether neurons are isopotential under different conditions. Current was injected at one or more locations in the model, and membrane potentials throughout the different compartments were measured. To investigate the behavior of pyloric neurons under free-running conditions, the spread of postsynaptic potentials was simulated, with synaptic currents injected at appropriate anatomical locations in the dendritic arbor. To consider how action potentials might influence membrane potentials in the dendritic arbor, the spread of brief current pulses injected in the primary neurite was simulated. Finally, to investigate the response of pyloric neurons under voltage clamp, current was injected at the soma; the results were compared to the results obtained from an equivalent cylinder model.
Materials and Methods

Electrophysiology and Dye Fills

Panulirus interruptus were purchased from Don Tomlinson Commercial Fishing (San Diego, CA, USA), and maintained in aquaria at 10-13C with circulating artificial seawater. Dissection was standard (Selverston et al., 1976). Preparations were perfused with 11-13C P. interruptus saline (479 NaCl, 12.8 KCl, 13.7 CaCl$_2$, 3.9 Na$_2$SO$_4$, 10 MgSO$_4$, 11.1 Tris, 5.1 maleic acid, all in mM; pH 7.5-7.6; Fisher Scientific, Pittsburgh, PA, USA). Extracellular recordings from lateral and medial ventricular nerves were made using bipolar stainless steel electrodes insulated with petroleum jelly and an A-M Systems differential amplifier (Carlsberg, WA, USA). Intracellular recordings were made using sharp glass microelectrodes filled with 2.5 M KAc, 20 mM KCl (resistance 10-20 MΩ) and an Axoclamp 2B (Sunnyvale, CA, USA). Pyloric neurons were identified by comparing intracellular and extracellular activities (Selverston et al., 1976).

Neurons were filled with Alexa Fluor 568 hydrazide sodium salt (10mM) or Alexa Fluor 555 hydrazide tris salt (10mM) (Invitrogen, Carlsbad, CA, USA). Microelectrodes were backfilled with dye by capillary action, and dye was injected by capacitance overcompensation on the Axoclamp. When the soma was deeply stained, capacitance was recompensated, and -2 to -5 nA current was injected for 10-20 min, followed by an additional 10 min diffusion with the electrode removed. The ganglion was then removed from the stomatogastric nervous system and fixed in 4% paraformaldehyde (Electron Microscopy Services, Hatfield, PA, USA), followed by an ethanol dehydration series (50, 70, 95, 100, 100%). The ganglion was then mounted on a glass microscope slide with a 0.12 mm spacer (Invitrogen) and cleared with methyl salicylate, and the coverslip sealed with nail polish. Ganglia were stored at 4C until viewing.
**Confocal microscopy**

Ganglia were viewed with a Zeiss LSM 510 confocal laser scanning microscope with oil immersion objectives, 40x Plan-NeoFluar, NA = 1.30, or 100x alpha-Fluar, NA = 1.45. Excitation wavelength was 543 nm, with an HFT477/543 primary beam splitter used. A 560 nm lowpass filter or 560-615 nm bandpass filter was used for Alexa 568 or 555, respectively. XY slices were digitized at 1024 pixels by 1024 pixels of 0.32 x 0.32 μm. Slices were taken with a 1 μm Z step size, sufficient to digitize the dendritic tree. The top of the soma was removed where necessary to allow for sufficient Z travel of the microscope to scan the entire dendritic tree. Due to the magnification used, it was impossible to digitize the entire dendritic tree in one stack. Instead, scanning was performed in several overlapping sections. The image stacks were exported as TIFF files, and then assembled into three-dimensional section files.

To merge the multiple section files into a single three-dimensional image, the individual section files had to be aligned against each other. However, due to the size of these section files it was not feasible to align them by simply adjusting the full images until they overlapped. Instead, each section file was first filtered using the Visualization Toolkit (vtk.org) to identify contour isosurfaces, the three-dimensional equivalent of contour isolines which identify the boundaries of a region of a given altitude in a map. These isosurfaces thus identified the boundaries of regions with high intensity, typically the dye-filled neural tree. The isosurfaces were then aligned against each other until they overlapped maximally. Rough alignment was performed manually, using the open source paraview program (paraview.org). Fine alignment was done using the Nelder-Mead simplex optimizer (described in *Chapter 3 – Materials and Methods - Assessing pathfinding performance*) (Nelder JA and Mead R, 1965), which further adjusted the position and rotation of one section file to maximize overlap with the other. The section files were then combined into a full image file.
Multi-scale vesselness calculation

The semiautomatic tracing algorithm functioned by following the center of dendrites. For very small dendrites, the center was readily identifiable as a thin bright region; only those voxels (three-dimensional pixels) within this region had high intensity values, and adjacent voxels were lower in intensity. For larger dendrites, however, many adjacent voxels typically had the same or similar intensity values, so simply following the path of highest intensity did not trace the center of the dendrite. Additionally, the confocal images contained noise and other artifacts which could impede the semiautomatic tracing process by providing spurious alternate paths for the trace to follow.

To minimize the influence of noise and enhance dendrite contrast, the three-dimensional images were processed to calculate each voxel’s vesselness, a scalar value which describes the degree to which it and its surrounding region are “tube-like” (Antiga L, 2007; Frangi AF et al., 1998). Vesselness is calculated at a particular scale, $\sigma$, governing the size of features detected. First, the image was smoothed by convolving with a Gaussian kernel of size $\sigma$, also known as Gaussian blurring. Then, the Hessian matrix at each voxel was calculated using data from that voxel and its neighbors. The Hessian matrix is the matrix of second-order partial derivatives of the image intensities, i.e.,

$$H(v) = \begin{bmatrix}
\frac{\partial^2 v}{\partial x^2} & \frac{\partial^2 v}{\partial x \partial y} & \frac{\partial^2 v}{\partial x \partial z} \\
\frac{\partial^2 v}{\partial y \partial x} & \frac{\partial^2 v}{\partial y^2} & \frac{\partial^2 v}{\partial y \partial z} \\
\frac{\partial^2 v}{\partial z \partial x} & \frac{\partial^2 v}{\partial z \partial y} & \frac{\partial^2 v}{\partial z^2}
\end{bmatrix}$$

where $v(x,y,z)$ is the voxel intensity (value). The Hessian thus describes the local curvature of the image intensity. The size of the Gaussian kernel, $\sigma$, thus governed the scale over which the vesselness calculation was made.
Then, the three eigenvalues of the Hessian matrix were calculated at each voxel, identifying the magnitude of the three orthogonal eigenvectors describing the local gradient. These eigenvalues, $\lambda_1$, $\lambda_2$, and $\lambda_3$, were sorted by increasing absolute magnitude, i.e., $|\lambda_1| \leq |\lambda_2| \leq |\lambda_3|$. These eigenvalues indicate the shape of the region. For example, in the center of a bright disk-like region, image intensity drops off rapidly only in one direction, so $\lambda_3 << 0$ and $\lambda_2 = \lambda_1 = 0$.

Tube-like regions have $\lambda_3 << 0$, $\lambda_2 << 0$, and $\lambda_1 = 0$ (Sato et al., 1998) Intuitively, regions are identified as tube-like because the image intensity does not change along the axial direction, but drops rapidly when moving radially (Figure 83).

![Figure 83. Eigenvalues for shape detection in vesselness calculation.](image)

A Hessian matrix is calculated over a particular scale (length of arrows) to determine the local curvature of image intensity. Its eigenvectors (red, green, and blue) identify the principle directions of gradient. In a bright disk-like region on a dark background (A), there is no radial gradient (blue, green), so the eigenvalues ($\lambda$) are 0. There is an axial gradient (red); voxel intensity rapidly decreases at the boundary (thick red arrow), so $\lambda_3 << 0$. In a tube-like region (B), with no axial gradient (red), $\lambda_1 = 0$. Radial gradients exist (green, blue), so $\lambda_2 << 0$ and $\lambda_3 < 0$. 

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Three factors were then calculated from the eigenvalues:

\[ R_A = \frac{|\lambda_2|}{|\lambda_3|} \]

which has value close to 1 for tube-like regions,

\[ R_B = \frac{|\lambda_3|}{|\lambda_2\lambda_3|} \]

which has value close to 1 for disk-like regions, and

\[ S = \sqrt{\lambda_1^2 + \lambda_2^2 + \lambda_3^2} \]

used to control sensitivity to noise. Vesselness was then calculated from these factors to identify bright features on a dark background:

\[ V(x) = \begin{cases} 
1 - e^{-\frac{R_A^2}{2\alpha^2}} & \text{if } (1 - e^{-\frac{R_A^2}{2\beta^2}}) \left(1 - e^{-\frac{s^2}{2\gamma^2}}\right) \lambda_1 < 0 \text{ and } \lambda_2 < 0 \\
0 & \text{otherwise}
\end{cases} \]

where \( \alpha, \beta, \) and \( \gamma \) are tuning parameters, e.g., 0.5, 0.5, and 0.25 (Frangi AF et al., 1998).

Vesselness calculation was repeated on multiple scales, by varying the \( \sigma \) of the Gaussian blurring kernel, to identify tube-like regions of varying sizes. Each voxel’s multiscale vesselness was calculated as the maximum of all vesselnesses, i.e.,

\[ V_{ms}(x) = \max(V_{\sigma_1}(x), V_{\sigma_2}(x), \ldots) \]

A new image was then constructed where voxel intensity was set to this multiscale vesselness value. Tube-like regions were clearly identified by this process (Figure 84). Vesselness calculations were performed using custom software written in C++ utilizing the open source Visualization Toolkit (vtk) and Insight Segmentation and Registration Toolkit (itk) (Kitware, Inc; vtk.org) (Schroeder et al., 2006); \( \sigma \) ranged from 1 to 16 in 8 logarithmic steps. To reduce the amount of memory required, vesselness calculation was performed in split regions of the image, with overlap of 3 \( \sigma \).
Semiautomatic tracing

Tracing of neural processes was performed using the **InteractiveTrace** program, custom software written in C++ utilizing **vtk** and **itk**. The tracing program loaded the original confocal image and multi-scale vesselness image and then built a “skeleton” or tree structure of coordinates following the center of the processes. The operator selected the first point, typically

*Figure 84. Confocal image before and after vesselness calculation*

Three-dimensional confocal image (*left*) was processed by detecting *vesselness*, a measure of how “tube-like” a region is. A new image (*right*) was constructed with each voxel’s intensity set to its vesselness value. Images are colored according to intensity (*white – red – blue – black*). Note the presence of noise and discontinuities on the left, absent in the vesselness image. The contrast of these images is high to improve visibility of the interior of the dendrites; accordingly, some small dendrites may not be visible.

Semiautomatic tracing

Tracing of neural processes was performed using the **InteractiveTrace** program, custom software written in C++ utilizing **vtk** and **itk**. The tracing program loaded the original confocal image and multi-scale vesselness image and then built a “skeleton” or tree structure of coordinates following the center of the processes. The operator selected the first point, typically
within the soma. To extend a tree, the operator selected a new point somewhere within the center of a dendrite. The program then followed the center of the dendrite back to the previous point (Figure 85).

![Figure 85. The InteractiveTrace semiautomatic tracing program](image)

Tracing began by identifying a point within the soma (not shown). Then, a point was chosen within a neural process, and the program traced back to the previous point (in the soma), by descending the last point’s Fast Marching gradient. A new point was suggested automatically using the Fast Marching method (see text, below). Branches were inserted manually. Original confocal images (left) and vesselness images (right) are shown in early (top) and later (bottom) stages of tracing. The dendrite skeleton following the center lines of the dendrites is shown in green.
To trace back from a new point to the previous endpoint, the tracing program made use of
the Fast Marching Method algorithm (Sethian, 1996) on the vesselness image. The Fast Marching
Method, analogous to Dijkstra’s algorithm, calculates how a wavefront propagates through
regions of low or high permeability; in this case, permeability was image intensity. Intuitively,
one can think of the algorithm as simulating a spreading pool of acid which dissolves more
rapidly through the brighter areas of the image, tagging each point with the time when it
dissolved.

The algorithm begins with an “already explored” set, $S_{\text{frozen}}$, containing the starting point
(the previous traced endpoint), and a “wavefront” set $S_{\text{narrowband}}$ of all its immediate neighbors.
Each initial point in $S_{\text{narrowband}}$ is assigned a “travel time” $t(p)$ equal to the distance from the initial
point, divided by its image intensity. The algorithm iterates by finding the wavefront point $p_{\text{min}}$
with the lowest travel time, and adding it to $S_{\text{frozen}}$. Then the unexplored neighbor $p_{\text{adj}}$ of $p_{\text{min}}$ each
become part of the wavefront, each assigned a travel time $t(p_{\text{adj}})$ combining its image intensity
and the travel time of its explored neighbors (for details see (Sethian, 1996)). The wavefront
eventually propagates throughout the entire image, with the travel time at each point reflecting
the time at which the wavefront passed. This produces a travel time gradient which descends
towards the origin point along the path of highest image intensity.

Tracing back from the new point to an old point in the dendrite skeleton was thus
accomplished by calculating a Fast Marching gradient from the old point, and then descending
that gradient from the new point, repeatedly moving to the adjacent point with lowest travel time.
After a new point was added to the dendrite skeleton, the InteractiveTrace program identified a
next suggested point. This was done by repeating the Fast Marching Method, starting this time
from the newly added point. The points on the surface of a sphere (of user-configurable size)
centered at the new trace were sampled. The sphere boundary point with the lowest Fast Marching gradient was chosen; this was the location with the lowest cost path back to the newly added point. To prevent doubling back, only the region of the sphere within $2\pi$ of the current direction was sampled.

To allow for tracing of large or densely illuminated neurites, in which a poor vesselness gradient existed, the fast marching algorithm was instead performed using the original image data instead of the vesselness values. In terminal regions where neither the vesselness data nor the confocal image provided a reliable gradient, manual routing was occasionally performed. To improve rendering performance, only voxels in a clipped box around the region being traced were typically displayed. Either the original confocal images or the vesselness images could be viewed during tracing. Clip box size, palette, visual contrast, color map, step size, and other parameters were manually adjusted during tracing for optimal performance and utility.

**Converting dendritic skeletons to volume models**

Once tracing was completed for a neuron, the result was a tree structure that faithfully followed the center lines of the neural processes. These were automatically converted to volume models. Because the confocal image quality and intensity values varied from image to image, there was no single intensity value which corresponded to the boundaries of the dendrites. Voxels on the outer edge of neural processes typically ranged in intensity value from 8 to 24; noise throughout the image had intensity values of 8 or less. Thus, contour isosurfaces of the original image data were calculated for voxel intensity values of 8, 12, 16, 20, and 24. If the intensity threshold was too high, then the contours would not identify the surfaces of the dendrites, but instead find interior regions. If the intensity threshold was too low, the contours would follow noise and artifacts in the image.
Starting with the intensity 8 contour isosurface, every point along the dendrite skeleton was assigned a radius by measuring the distance from it to the nearest contour isosurface (note that distance between adjacent points along a skeletal trace was typically close to the spacing between voxels). This was repeated for each other intensity level contour isosurface. To identify the correct contour level, each “fleshed out” skeleton was tested to determine whether the radii of the dendrites dropped appropriately with increasing path distance, i.e., distance along the tree from the soma. Visualization of radius versus path distance for various traces suggested a power law relationship, thus, testing was done by fitting a power law model to the radii \( r \) and path distance \( d_p \):

\[
r = a \cdot d_p^b
\]

The contour isosurface whose power model gave the best fit, as indicated by the lowest residual standard error, was selected, with visual confirmation. The radii were then smoothed with 1000 passes of mean filtering (i.e., averaging each value with its adjacent neighbors); to prevent discontinuities at branch points, the radii at branch points were smoothed with all three adjacent radii. The coordinates and smoothed radius data were then converted into a set of connected cylindrical compartments for subsequent analysis utilizing the NEURON simulation environment (Hines and Carnevale, 2001), with one anatomical compartment per contiguous, unbranched section of the traced neuron, maintaining the full \( x, y, z \), and radius coordinates. The soma was converted to a single cylindrical compartment with one electrical segment, with a surface area equal to the spherical soma from the confocal image. When the soma was not present in the confocal image, previously established soma radii for the neuron type were used (Thuma et al., 2009). The number of segments (electrical compartments) per anatomical compartment was set using the \( d_\lambda \) rule (Hines and Carnevale, 2001), which provides an
estimate for how many compartments are needed to provide accurate results given the membrane properties and the frequency of perturbation. The number of segments was recalculated whenever any compartment’s membrane capacitance was changed. Neuron traces which were visibly truncated or which were of poor quality were excluded from subsequent analysis. Each electrical compartment was assigned a leak conductance and membrane capacitance.

*Positioning current sources*

To evaluate the range of membrane potentials which occurred with different types of current injection into the neuron, current sources were introduced into the passive models. To simulate synaptic currents, potential synapse locations were identified on dendrites with radius between 0.75 and 1.25 μm; this corresponded with previously established pyloric synapse locations (Cabirol-Pol et al., 2002). At each potential location, the path distance \( d_p \) to the soma was measured. The mean of all such path distances was calculated. The three potential locations with path distance to the soma closest to this mean were selected, to improve the likelihood that the three synapses would be roughly the same electrotonic distance from the soma. Multiple locations on the same branch were excluded. Constant current injection point processes, \( i_{syn1} \), \( i_{syn2} \), and \( i_{syn3} \) were then placed into the NEURON data file at the three identified locations.

To simulate the propagation of action potentials initiated in the axon, a current injection process, \( i_{axon} \), was placed in the proximal third of the primary neurite, the largest diameter branch exiting the ganglion. VD neurons were not used, as their axon bifurcates, with two spike initiation zones distal to the bifurcation (Thuma et al., 2009). To simulate the propagation of voltage clamp current, a somatic current injection point \( i_{soma} \) was placed in the soma. During simulations, only one type of current source (synaptic, axonal, or somatic) was active.
**Extrapolated anatomical models**

Because of the limitations of the tracing process, the anatomical models underestimated the total membrane surface area. The first method used to correct this was extrapolating the branching pattern by adding compartments to the model until the desired total membrane capacitance was reached. Membrane capacitance was used as an estimate of surface area, as available evidence indicates that specific membrane capacitance is a constant, 0.9 µF/cm\(^2\) (Gentet et al., 2000). At every point in the dendritic tree, the branch order, i.e., the number of branches which had occurred since the soma, \(n_{\text{branch}}\) was measured. Branch order was found to increase linearly with path distance \(d_p\) (Figure 86). Thus, a linear model, \(n_{\text{branch}} = m \cdot d_p + b\), was fit to the data; significant fits were obtained for all neuron traces (p<0.01).

![Figure 86](image)

*Figure 86.* Branch order versus path distance in neuron PY2

For each section of the traced neuron, the number of branches since the soma \(n_{\text{branch}}\) and distance along the path from the soma \(d_p\) were measured. A histogram was constructed, using two passes of mean filtering. In all neurons, a linear model, \(n_{\text{branch}} = m \cdot d_p + b\), was fit to the data and used to extrapolate the branching pattern to construct the extrapolated anatomical models.
Repeatedly, until the desired total membrane capacitance was achieved, the terminal dendrite with the smallest \( d_p > 250 \, \mu \text{m} \) was selected. If the branch order at the end of that dendrite was less than predicted by the branching model, a branch was added. Otherwise, the dendrite was simply extended until the next predicted branch point. When dendrites were added or extended, their radii were set as predicted from the previously fit power law model (*Materials and Methods – Converting dendritic skeletons to volume models*). Diameters of extrapolated branches were never less than 0.9 \( \mu \text{m} \). The resulting extrapolated anatomical models had multiple balanced tree-like extensions on the intermediate length dendrites (*Figure 87*). Specific capacitance (\( C_m \)) was set to 0.9 \( \mu \text{F/cm}^2 \) in every compartment, and specific conductance (\( G_m \), in \( \text{mS/cm}^2 \)) was set to \( G_0 \), a base conductance value adjusted later to provide a desired input resistance.

*Figure 87.* Original and extrapolated anatomical neuron model

The original trace of an LP neuron (A) was extrapolated by adding additional dendrites following the neuron’s dendritic radius and branching patterns (B). The soma is visible as a large cylindrical compartment (black rectangle)
Capacitance adjusted models

The second method used to correct for insufficient surface area was simulating the missing surface area electrically. This was done by increasing the conductance and capacitance of the membrane. As it was not known where the untraced regions of the neuron were located, three different capacitance adjusted models were made from each uncorrected model. Three methods were used, differing in where the additional capacitance was located. In all methods, both specific membrane conductance and specific membrane capacitance were scaled equally in a compartment. In flat adjustment, conductance and capacitance were increased the same amount in each compartment in the neuron; this simulated conditions such as untraced compartments distributed equally throughout the neuron, or rough or crenulated membrane with larger than expected specific capacitance. In linear adjustment, the scaling factor increased linearly with path distance from the soma; this simulated untraced compartments which were more common with further distance from the soma, as might occur due to poor dye diffusion. In terminal adjustment, the scaling factor was high only in terminal regions, and low elsewhere; this simulated a large area of untraced membrane located only in distal areas. To achieve these adjustments, each compartment $c$ was first assigned a distality value $\varphi(c)$:

$$\varphi(c) = \frac{|T(c)| \cdot d_p(c)}{\sum_{i \in T(c)} d_p(i)}$$

where $T(c)$ are all the terminal compartments distal to $c$ (but not the intermediate branches), $|T(c)|$ is the count of those terminal compartments branching off of $c$, $d_p(c)$ is the path distance to $c$, and $d_p(i)$ is the path distance to terminal compartment $i$. In other words, distality was the path distance of the compartment divided by the mean path distance of all terminal compartments branching off of it, and thus ranged from 0 for the soma, to 1 at terminal compartments. The
specific membrane conductance \( G_m(c) \), in mS/cm\(^2\) and specific membrane capacitance \( C_m(c) \), in \( \mu F/cm^2 \) in each compartment \( c \) other than the soma was then set:

\[
G_m(c) = \begin{cases} 
G_0 & \text{flat adjustment} \\
G_0 \varphi(c) & \text{linear adjustment} \\
G_0 \varphi(c)^6 & \text{terminal adjustment}
\end{cases}
\]

\[
C_m(c) = \begin{cases} 
C_0 & \text{flat adjustment} \\
C_0 \varphi(c) & \text{linear adjustment} \\
C_0 \varphi(c)^6 & \text{terminal adjustment}
\end{cases}
\]

with \( G_0 \) and \( C_0 \) being base conductance and capacitance values. The large exponent \( (\varphi(c)^6) \) used in terminal adjustment ensured that \( \varphi(c) \) would be small in proximal regions, and large in terminal regions near the ends of the dendrites (large by comparison; note the scaling of \( G_0 \) and \( C_0 \) base values, below). The soma was assigned specific membrane conductance and capacitance of \( G_0 \) and \( C_0 \) respectively. These base values, \( G_0 \) and \( C_0 \), were then increased until the neuron model had the desired input resistance (see below) and total membrane capacitance.

Determining appropriate total capacitance

Electrical capacitance measurements were not available for the traced neurons, so approximate capacitance was estimated. For LP and PY neurons, mean capacitance was known from previous experiments (Chapter 5), 12 ± 3.6 nF, and 5.4 ± 1.7 nF respectively. The total membrane capacitance of the uncorrected LP and PY anatomical model neurons was measured, assuming a specific membrane capacitance of 0.9 uF/cm\(^2\) specific membrane capacitance (Gentet et al., 2000). These were divided by the electrophysiological capacitance measurements to estimate the fraction of membrane which was traced. Estimates ranged from 18% to 36% (see also Discussion). Extrapolated and capacitance adjusted models were then constructed from the uncorrected models, increasing total membrane capacitance to 5 times the uncorrected value, as a worst-case condition for evaluating isopotentiality.
**Input resistance at different membrane potentials**

During voltage clamp experiments in pyloric neurons (*Chapter 4*), it was noted that the input resistance ($R_N$) was substantially lower at more depolarized holding potentials. During voltage clamp experiments using the **cp1** perturbation (*Chapter 3*), while only moderate clamp current was observed during voltage clamp potentials close to $V_{rest}$, clamp current increased nonlinearly with increasingly depolarized clamp potentials, with clamp current of 200 nA or more observed during holding potentials near -5 mV (*Figure 88*). When voltage clamping pyloric neurons to fixed potentials to assess clamp quality, decreases of input resistance of ten-fold ($V_{clamp} = -30$ mV) or hundred-fold ($V_{clamp} = -5$ mV) were commonly observed. Similarly, in current clamp experiments, current injection during the depolarized phase of the spontaneous oscillation of pyloric neurons produced less membrane potential change than the same current injection during the hyperpolarized phase.

*Figure 88. Voltage clamp current increases substantially with depolarizing clamp voltages*

Voltage and current samples were obtained from voltage clamp perturbation experiments with a single neuron voltage clamped with the **cp1** composite perturbation (see *Chapter 3*)
Based upon these data, nominal input resistances, $R_{\text{nominal}}$, were selected to represent the input resistance of an average pyloric neuron under different conditions. For pyloric neurons at or near rest potential, $R_{\text{nominal}}$ of 10 MΩ and 5 MΩ were used; this accounted for the range of input resistances observed in pyloric neurons at or near rest potential, even in neurons which may have been substantially undertraced. To simulate the spread of current at a membrane potential or voltage clamp command potential near -30 mV (the upper limit of the spike initiation voltage), $R_{\text{nominal}}$ of 1 MΩ was used. To assess current flow when voltage clamped to depolarized potentials near -5 mV, the most depolarized potential used in the perturbations (see Chapter 3), $R_{\text{nominal}}$ of 0.1 MΩ was used.

The different traced pyloric neurons had different total membrane surface area. The actual $R_N$ used for a given neuron was

$$R_N = \frac{\bar{A}}{A} \cdot R_{\text{nominal}}$$

Where $A$ is the neuron membrane surface area, and $\bar{A}$ is the mean surface area of all traced neurons (mean surface area was calculated separately across the extrapolated models). Neurons with larger membrane surface area, and putatively greater total membrane conductance, thus had lower input resistance.

**Setting anatomical model input resistance**

The input resistance ($R_N$) of each capacitance adjusted and extrapolated model was set to different values throughout the experiment. For example, in simulated voltage clamp conditions with a clamp potential of -5 mV, the input resistance was set to $\bar{A}/A \cdot 0.1 \ \text{M}\Omega$, i.e., a nominal input resistance of 0.1 MΩ scaled in inverse proportion to the neuron’s actual membrane surface area. Setting a specific input resistance in the model was achieved by adjusting the membrane properties until measured input resistance equaled the desired input resistance. The base specific
conductance \((G_0)\) was set to 1 mS/cm\(^2\), and 1 nA was injected in the soma. The soma membrane potential change was measured after 1000 ms, and \(R_N\) was calculated using Ohm’s law. \(G_0\) was then adjusted using a binary search, increasing or decreasing in smaller steps, until the measured \(R_N\) was within 5% of the desired \(R_N\). Base capacitance was not changed when base conductance was adjusted. Note that this altered the time constant \((\tau = R_mC_m)\) of each compartment, however, it was expected that the time constants would change in a real neuron as input resistance changed, due to voltage-dependent conductances contributing to \(R_m\).

**Setting current injection amplitudes**

The amplitude of the three synaptic currents, \(i_{syn1}\), \(i_{syn2}\), \(i_{syn3}\), were set in each extrapolated or adjusted model by binary search until a membrane potential deflection of -4 mV was achieved in the soma after 25 ms. This corresponded with typical features of inhibitory post-synaptic potentials in pyloric neurons (*Figure 8*9). Adjustment was performed with the \(R_N\) of the model set to 10 M\(\Omega\), typical at the hyperpolarized membrane potentials during which IPSPs appear in pyloric neurons. The axonal injected current \(i_{axon}\) was adjusted similarly, until membrane potential deflection of 15 mV, a typical amplitude of fast sodium spikes observed from the soma, was achieved in the soma 5 ms after current injection with \(R_N = 1\ M\Omega\). Input resistance was reduced to correspond to the \(R_N\) at the depolarized phase during which spikes occurred (*Figure 89*). For all adjustments, a constant integrator time step of 1 ms was used. Note that these synapses were simple current sources and did not have a reversal potential, thus, they were capable of hyperpolarizing regions of the dendrite below \(E_{Cl}\) or \(E_K\).
Measuring membrane potential distribution in response to current injection

Current was injected into the capacitance adjusted and extrapolated models to assess the range of membrane potentials produced throughout the neuron. Three different tests were conducted. In the first, inhibitory post-synaptic potentials (IPSPs) were simulated with 25 ms of constant hyperpolarizing current injected at the three dendritic synapses, using the previously calculated current values. In the second, action potential propagation was simulated with 4 ms of constant depolarizing current injected at the axon, again using the previously determined current value. Finally, voltage clamp was simulated by injecting depolarizing current in the soma for 1 s, and adjusting the current amplitude until the soma was depolarized or hyperpolarized to the command potential. This reflected the conditions of actual voltage clamp in real neurons, insofar as current is both measured and injected only in the soma.

Equivalent cylinder models

The membrane potentials achieved in the passive neuron models in response to voltage clamp were compared with the predictions of equivalent cylinder models. In principle, a dendritic arbor can be simplified to an equivalent cylinder provided certain assumptions hold true,
in particular that all terminal branches have the same electrotonic distance from the trunk, and that the dendrite diameters at a branch follow the $d^{3/2}$ rule, i.e., when dendrite $a$ branches into $b$ and $c$, then $d_a^{3/2} = d_b^{3/2} + d_c^{3/2}$ (Rall, 1962). These criteria are not commonly satisfied in real neurons, and neither was satisfied in the traced pyloric neurons. Instead, equivalent cylinder models were constructed based upon time constants of multi-exponential fits obtained during current clamp capacitance measurements of PY and LP neurons (Chapter 5). Discounting the slowest exponential term’s time constant $\tau_0$ which was due to a residual voltage-dependent phenomenon, the next two time constants were used to estimate an electrotonic length $L$ for an equivalent cylinder model of the neuron:

$$L = \frac{\pi}{\sqrt{\tau_1/\tau_2 - 1}}$$

where $\tau_1$ is coefficient representing the passive membrane time constant, and $\tau_2$ is the first of the equalizing time constant coefficients (Rall, 1969). A lower and upper estimate of electrotonic lengths were thus obtained from neurons having input resistances between 4 and 10 MΩ, with measurements made at membrane potentials between -50 and -80 mV. These estimates were used to calculate the membrane potentials throughout equivalent cylinders voltage clamped from $V_{rest} = -65$ mV to $V_{clamp} = -55$ mV:

$$V(X) = V_{rest} + (V_{clamp} - V_{rest}) \cdot \frac{\cosh(L - X)}{\cosh(L)}$$

where $V_{rest} = -65$ mV, $V_{clamp}$ is the clamp potential, and $X$ is the electrotonic distance ranging between 0 (at the proximal end) and $L$ (at the distal end).

To construct equivalent cylinder models to represent voltage clamping to potentials of -30 mV or -5 mV, during which input resistance was 10-fold or 100-fold lower respectively, the electrotonic length $L$ of the equivalent cylinders were increased. Note that $L = l/\lambda$, where $l$ is the
length of the cylinder, and \( \lambda \) is the length constant, calculated as follows (discounting extracellular resistance):

\[
\lambda = \sqrt{\frac{R_m d}{4 R_i}}
\]

where \( R_m \) is the specific membrane resistance in \( \Omega \) cm\(^2\) (assumed to be uniform), \( d \) is the diameter, and \( R_i \) is the intracellular axial resistance in \( \Omega \) cm. Thus, \( \lambda \) decreases, and \( L \) increases, with the square root of membrane resistance. However, the relationship between input resistance and membrane resistance depends upon the geometry of the cylinder. The input resistance of an infinite cable is given by

\[
R_\infty = \lambda \cdot \frac{4R_i}{\pi d^2}
\]

and the input resistance of a finite, open-ended cable by

\[
R_N = R_\infty \cdot \coth(l/\lambda)
\]

(Rall and Rinzel J., 1973). While a ten-fold decrease in \( R_m \) will thus produce a 3.16 times decrease in \( R_\infty \), the effect upon \( R_N \) is not straightforward, depending upon the values of \( l \) and \( d \).

To estimate approximate scaling factors for \( R_m \) for 10-fold and 100-fold changes in \( R_N \), the specific membrane conductance values obtained when setting \( R_N \) to target values in traced neurons with flat capacitance adjustment (see Results, below) were used. For each traced neuron, the two scaling factors were calculated:

\[
\rho_{10} = \frac{G_m(R_{\text{nominal}} = 1M\Omega)}{G_m(R_{\text{nominal}} = 10M\Omega)}
\]

\[
\rho_{100} = \frac{G_m(R_{\text{nominal}} = 0.1M\Omega)}{G_m(R_{\text{nominal}} = 10M\Omega)}
\]

For voltage clamping to -30 mV, with \( R_N = 1M\Omega \), and to -5 mV at \( R_N = 0.1M\Omega \), electrotonic length was thus scaled as follows:
\[ L(-30mV) = L \cdot \sqrt{\rho_{10}} \]
\[ L(-5mV) = L \cdot \sqrt{\rho_{100}} \]

with \( \rho_{10} \) and \( \rho_{100} \) being the mean of these scaling factors across all traced neurons. Voltage drop along these equivalent cylinders was calculated as above.

**Visualization and statistical analysis**

In each test, the membrane potential in each compartment at the end of the current injection was written to a file, along with the compartment membrane surface area. Weighted quantiles were then calculated using the `wtd.quantile` function of the `Hmisc` package of the open source statistical computation language R (r-project.org). The mean, median, and 0.5%, 2.5%, 5%, 95%, 97.5%, and 99.5% area-weighted quantile potentials were calculated. For example, if the 5% quantile potential was -90 mV, then only 5% of the membrane (weighted by surface area) was below -90 mV, and 95% was above it. The membrane potential range across 90% of the neuron, surface, \( V_{90} \), was calculated as the difference of the 5% and 95% quantile points. These quantile points were designated \( V_{90H} \) (the hyperpolarized end of the range) and \( V_{90D} \) (the depolarized end) respectively. For example, if the 5% quantile point \( (V_{90H}) \) was -77 mV, and the 95% quantile point \( (V_{90D}) \) was -63 mV, then \( V_{90} \) was 14 mV, i.e., 90% of the neuron membrane surface area was within 14 mV. \( V_{95} \) (95% range) and \( V_{99} \) (99% range) and depolarized and hyperpolarized endpoints were calculated similarly. These analyses were often conducted by considering the different capacitance adjustment models as different classes, e.g., considering whether the extrapolated models were significantly less affected by current injection than the adjusted models.

Statistical tests were conducted using Student's t-tests or single- or multi-factor ANOVA as indicated, with post-hoc tests performed with Tukey's Honest Significant Differences test.
Statistical tests were conducted using R. Where multiple comparisons were made with the same source data, p-values were adjusted via the Holm-Bonferroni method (Aickin and Gensler, 1996); reported p-values are the adjusted values. Multiple exponential fits were performed on the charge curves of some neurons using the multiple run fitter of gnuplot (gnuplot.info). NEURON shape plots were made of membrane potentials throughout the models at the end of each current injection. Additional plots were made using gnuplot or the native graphing facilities of NEURON.
Results

Tracing

A total of 13 neurons (3 IC, 2 LP, 3 PD, 2 PY, 3 VD) were traced with sufficient fidelity and detail for subsequent analysis. Four neurons were excluded due to insufficient dye penetration or poor confocal image quality. AB neurons could not be successfully traced due to poor image contrast, possibly due to dye leakage. Tracing with the InteractiveTrace program was rapid, yielding highly detailed anatomical models (Figure 85, Figure 90). The resulting models displayed occasional radius errors, e.g., nodular regions on terminal processes where the contour isosurfaces were inaccurately calculated due to visual noise. Manually removing these artifacts did not affect results. Diameters were almost always above 0.1 µm, with only a few fine terminal processes (<0.1%) having smaller diameters (Figure 91).

Figure 90. NEURON model from a traced VD neuron

The VD neuron is morphologically unique in having two axons (large neural processes exiting to the left). Occasional fluctuations in radius occurred (e.g. on the upper axon).
Traced neuron capacitance, surface area, and resistance

Surface area of the traced neurons ranged from $0.95 \cdot 10^{-3}$ cm$^2$ (PY1) to $3.26 \cdot 10^{-3}$ cm$^2$ (VD2), with a mean of $2.29 \cdot 10^{-3}$ cm$^2$. Capacitance was calculated for all traced neurons, assuming a specific membrane capacitance of 0.9 µF/cm$^2$ (Table 15). No electrophysiological measurements were available from these neurons, so capacitance could not be measured based upon the membrane response to current steps. However, capacitance measurements of other LP and PY neurons were available from previous experiments (Chapter 5). Among LP and PY neurons, the capacitances of the traced neurons were 2.75 to 5.5 times smaller than the electrophysiological measurements. This suggested that the neurons were incompletely traced. Extrapolated models and capacitance adjusted models were thus constructed from the original traced models, to increase the membrane capacitance by 5x. This large but possibly realistic value was chosen to consider a worst case condition for evaluating isopotentiality.
Table 15

Neuron Area, Capacitance, and Specific Capacitance by Adjustment Method

<table>
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<tr>
<th>Cell</th>
<th>Area</th>
<th>Orig C</th>
<th>Flat C</th>
<th>Linear C&lt;sub&gt;m&lt;/sub&gt;</th>
<th>Terminal C&lt;sub&gt;m&lt;/sub&gt;</th>
<th>Extr C</th>
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<td>mm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>C&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Soma Min</td>
<td>Max</td>
<td>Mean</td>
<td>Soma Min</td>
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<td>0.246</td>
<td>2.22</td>
<td>6.04</td>
<td>1.81</td>
<td>6.04</td>
<td>4.76±0.95</td>
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<td>IC2</td>
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</table>

Orig C: Original traced neuron capacitance (nF) \((C_m = 0.9 \mu F/cm^2)\). Flat \(C_m\): specific membrane capacitance \((\mu F/cm^2)\) in flat adjusted model. Linear/Terminal \(C_m\): specific membrane capacitance in soma, and minimum, maximum, and mean ± standard deviation (area weighted) in dendritic compartments, in linear and terminal adjusted model. Extr C: neuron capacitance (nF) in extrapolated model \((C_m = 0.9 \mu F/cm^2)\).
Input resistance of each adjusted and extrapolated model was then set by a binary search during which $G_0$, the specific membrane conductance in the soma, was set and input resistance calculated, adjusting $G_0$ as needed. Whenever $G_0$ was changed, $G_m$ in each other compartment was recalculated as appropriate for the compensation method. In the flat adjustment model, in which $G_m = G_0$ in every compartment, mean $G_m$ ($R_{\text{nominal}} = 5 \text{ M}\Omega$) was 88.9 µS/cm². One neuron (PY2) had a high $G_m$, 127 µS/cm² ($R_{\text{nominal}} = 5 \text{ M}\Omega$), suggesting impaired propagation of current. However, this neuron was extensively traced and had no obvious visible artifacts or defects.

Table 16

Neuron Input Resistance and Membrane Conductance by Adjustment Method

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<th>Flat $G_m$ Soma Min Max Mean</th>
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<td>41.5 52.8 15.8 52.8 41.6±8.30</td>
<td>114 0.1 114 41.3±34.7</td>
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<td>(5)</td>
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Cell: rows showing neuron number (e.g., IC1) are values for nominal input resistance ($R_{\text{nominal}}$) = 10MΩ; next three rows show values for $R_{\text{nominal}}$ = 5, 1, and 0.1 MΩ. $R_N$: actual input resistance of neuron. Flat $G_m$: specific conductance ($\mu$S/cm$^2$) throughout neuron with flat adjustment method. Linear/Terminal $G_m$: specific conductance in soma, and min, max, and mean ± standard deviation in dendritic compartments (area weighted) in linear and terminal adjustment methods. Extr $G_m$: membrane specific conductance ($\mu$S/cm$^2$) throughout neuron in extrapolated models.

*Simulated inhibitory post-synaptic potentials*

IPSPs were simulated using the three constant current synapses. These were located according to previous research showing that pyloric neurons have very few close appositions located on more proximal processes (Cabirol-Pol et al., 2002). IPSPs of 2 - 4 mV and 25 ms are typically observed in pyloric neurons (Figure 89). An IPSP size of 4 mV was selected as a worst case scenario for assessing isopotentiality. At a nominal input resistance of 10 MΩ, individual neuron model $R_N$ ranged from 7 to 24 MΩ. At a nominal input resistance of 5 MΩ, neuron input resistance ranged from 3.5 to 12 MΩ (synaptic current was recalculated when nominal input resistance was changed). At either nominal input resistance, even the more detailed, and thus presumably less isopotential, models exhibited a tight range of membrane potentials throughout the bulk of the neuron (Figure 92), excepting the isolated regions distal to the synapses (the model did not constrain membrane potentials in these distal regions by the synaptic current’s reversal potential, however, this only occurred in one neuron). The extrapolated models were
generally less isopotential, with regions remaining at $V_{rest}$, especially when the additional membrane surface area was added to a few electrically distal processes instead of being distributed widely throughout the dendritic arbor (*Figure 93*).

The $V_{90}$, $V_{95}$, and $V_{99}$ membrane voltage ranges were then calculated (e.g., if the $V_{90}$ was -70 to -77 mV, this means that 90% of the neuron membrane surface was within this 7 mV range). These were visualized as whisker plots (*Figure 94*). At either nominal input resistance, the depolarized end of the ranges, representing the portions of the neurons which were less adequately reached by the synaptic currents, differed significantly by area compensation type (ANOVA) with the larger regions of extrapolated models being significantly closer to the -65 mV resting potential than any of the corresponding capacitance adjusted models (Tukey’s Honest Significant Differences post-hoc test) ($V_{90D}$, $V_{95D}$: p<0.001; $V_{99D}$: p<0.05; both 5 and 10 MΩ nominal). No measure ($V_{90}$, $V_{95}$, $V_{99}$, or the depolarized or hyperpolarized ends thereof) varied significantly among the three capacitance adjustment model (ANOVA), though visualizations suggested the terminal capacitance method may have had more extensive penetration of IPSPs into the dendritic arbor (*Figure 96*). This is consistent with research demonstrating that non-uniform distribution of leak conductances with large amounts in distal regions increases propagation of synaptic currents (London et al 1999). When nominal $R_N$ was reduced from 10 MΩ to 1 MΩ or 0.1 MΩ, the hyperpolarization from the synaptic currents did not extend into the soma or the remaining processes in either the capacitance-adjusted or extrapolated models, instead being confined to the regions distal to the synapses (*Figure 95*)
Figure 92. Membrane potentials in adjusted and extrapolated PD2 neuron models in response to simulated IPSPs with $R_N = 8 \, \text{M}\Omega$ (10 M\(\Omega\) nominal)

IPSPs were injected into flat adjustment (A), linear adjustment (B), and terminal adjustment (C) models, and the extrapolated model (D), hyperpolarizing the neurons from the -65 mV resting potential (cyan). Compartments were colored according to membrane potential after 25 ms (scale, right). The models were generally isopotential.
Figure 93. Membrane potentials in adjusted and extrapolated PY2 neuron models in response to simulated IPSPs with $R_N = 5 \, \text{M}\Omega$ (10 M\Omega nominal)

Shown are responses after 25 ms to IPSPs in flat adjustment (A), linear adjustment (B), and terminal adjustment (C) models, and the extrapolated model (D). Regions distal to the synapses (yellow to magenta) are hyperpolarized below soma potential (green). In the extrapolated model made from this neuron, additional dendrites were added onto regions of the dendritic arbor remote from the synapses; accordingly much of the extrapolated membrane remains relatively depolarized, near resting potential of -65 mV (cyan).
Figure 94. Penetration of synaptic currents with $R_{\text{nominal}} = 10$ MΩ (A) and 5 MΩ (B), at 25 ms

For each neuron (label, x axis), compensation for untraced membrane surface area was made with four different methods. The four bars per neuron correspond (left to right) to these methods: flat capacitance adjusted, linear capacitance adjusted, terminal capacitance adjusted, and extrapolated models. The $V_{90}$, $V_{95}$, and $V_{99}$ ranges are shown as thick bars, thin bars, and whiskers. For example, at 10 MΩ (A), in all three adjusted models for IC2, 90% of membrane surface area ranged between -68 mV and -73 mV; 95% between -68mV and -74mV, and 99% between -66 mV and -85 mV. Black dots show soma membrane potential (-69 mV, as synaptic current was originally set to give -4 mV IPSPs in the soma); the dashed line at the top is the resting potential. IPSPs were not constrained by chloride or potassium reversal potentials.
Figure 95. Poor propagation of synaptic current with low input resistance.

As $R_N$ is decreased from 8 MΩ (A), to 0.8 MΩ (B) and 0.08 MΩ (C), 25 ms synaptic IPSPs are increasingly less effective at hyperpolarizing the neuron.
Figure 96. Deeper penetration of synaptic current in PY2 neuron with terminal than flat capacitance adjustment methods at $R_N = 5 \, \text{M}\Omega$

Red areas indicate regions of the PY2 neuron (compare Figure 93) hyperpolarized more by IPSPs in the terminal capacitance adjusted model than in the flat adjusted model. Black regions had similar membrane potentials. No regions were more hyperpolarized in the flat models.
Propagation of action potentials

Action potentials were simulated by injection of a brief (5 ms) current pulse in the primary neurite, using a nominal input resistance of 1 MΩ, a worst case scenario for input resistance during the depolarized phase of the slow wave oscillation. Current was adjusted until a soma depolarization of 15 mV was achieved after 4 ms. In contrast to the IPSPs, propagation of action potentials into the dendritic arbor was inconsistent (Figure 97), and many regions remained at relatively hyperpolarized potentials in individual neurons examined. These results were observed over all neurons (Figure 98), with some regions largely unaffected by the depolarizing current, and others driven to a potential more depolarized than the soma. The VDs were excluded from analysis because their axon bifurcates before the spike initiation zone (Thuma et al., 2009).

Excluding VD neurons, the extrapolated models differed significantly from the adjusted models, with large regions of the extrapolated neurons unaffected by the depolarization. In the extrapolated neurons, the hyperpolarized end of the ranges were less depolarized by the action potential ($V_{90H}$, $V_{95H}$, $V_{99H}$ $p<0.01$) and the depolarized end of the ranges were generally less depolarized ($V_{90D}$, $V_{95D}$: $p<0.01$; $V_{99D}$ not significant).
Figure 97. Response of adjusted and extrapolated models of PD2 neuron to simulated axonal action potential with $R_{\text{nominal}} = 1 \, \text{M}\Omega$.

Compare Figure 92. Measurements made at 4 ms.
Response to voltage clamp

To assess how voltage clamp current penetrates the dendritic arbor, constant current was injected in the soma for 100 ms to achieve a depolarization from $V_{\text{rest}} = -65 \text{ mV}$ to $V_{\text{clamp}} = -55 \text{ mV}, -30 \text{ mV},$ and $-5 \text{ mV},$ with $R_{\text{nominal}}$ set to 10 MΩ, 1 MΩ, and 0.1 MΩ, respectively. This corresponded to approximate input resistances observed in real neurons clamped to these potentials. Neurons were relatively isopotential only at the highest input resistance; at lower input resistances, extensive regions of the neurons had potentials ranging between $V_{\text{clamp}}$ and $V_{\text{rest}}$ (Figure 99). As input resistance was decreased, the $V_{90}$, $V_{95}$, and $V_{99}$, the range of membrane potentials across 90%, 95%, and 99% of the membrane, increased significantly ($p<0.05$). Despite the increasingly depolarized clamp command voltage, the hyperpolarized ends of the ranges ($V_{90\text{H}}, V_{95\text{H}},$ and $V_{99\text{H}}$) became significantly less depolarized (i.e., closer to $V_{\text{rest}}$), indicating that the neuron was less effectively clamped ($p < 0.001$, ANOVA with post-hoc test).

Figure 98. Response of all neurons at 4 ms to simulated action potential in axon

Compare to Figure 94. Membrane potentials in most neurons varied across a wide range, with many regions substantially more depolarized than the soma (black dots). The VD neurons, which have an axon that bifurcates proximal to the spike initiation zone, were excluded from analysis.

Response to voltage clamp
Surface area compensation method (flat, linear, terminal, or extrapolated) contributed to variations in results. A two-factor ANOVA was conducted for each of \( V_{90}, V_{90H}, V_{95}, V_{95H}, V_{99}, \) and \( V_{99H}, \) with \( R_N \) and surface area compensation method as factors. Nearly all had significant differences. Post-hoc tests confirmed that the hyperpolarized ends of the 90% and 95% ranges were significantly less well clamped in the extrapolated models than the adjusted models (\( p < 0.05 \)). The \( V_{90} \) and \( V_{95} \) ranges were significantly smaller in the extrapolated models, but at a potential closer to \( V_{rest} \) and further from \( V_{clamp} \), i.e., the neurons were less effectively clamped. A separate test was performed to determine whether the flat and terminal adjustment models differed. Paired t-tests conducted comparing the \( V_{90}, V_{95}, V_{99}, V_{90H}, V_{95H}, \) and \( V_{99H} \) confirmed that the terminal adjusted models were significantly more isopotential (i.e., a smaller voltage range) and significantly better clamped (i.e., the hyperpolarized end of the range was more depolarized) in all cases (all \( p < 0.05 \), adjusted for multiple comparisons with Holm-Bonferroni method).
Figure 99. Range of membrane potentials throughout neurons at 100 ms when voltage clamping to different soma potentials.

When voltage clamping to -55 mV, at which $R_N = 10 \text{ M}\Omega$ (*top*), neurons were relatively isopotential. When clamping to -30 mV, at which $R_N = 1 \text{ M}\Omega$ (*middle*), or to -5 mV, at which $R_N = 0.1 \text{ M}$ (*bottom*), much of the membrane surface area remained at or near $V_{\text{rest}}$ (-65 mV). Note different $y$ axis scales.
Time course of responses

Current propagated rapidly throughout the dendritic arbor in all models regardless of capacitance adjustment method (Figure 100). During the first few ms (e.g., Figure 100C), current equilibration among compartments occurred in many of the larger extrapolated models, as confirmed by multi-exponential fits. After this time, the entire dendritic arbor charged uniformly in all neurons and with all capacitance adjustment methods, with the ratio of membrane potentials among compartments remaining constant.

Figure 100. Time course of membrane potentials in response to current injection

Current was injected in the soma for 5 ms (A,C) or 100 ms (B,D) in flat (A,B) and extrapolated (C,D) models of neuron LP2, with \( R_N = 1 \, \text{M}\Omega \). Membrane potential was recorded in the soma (red), and in three distal locations (green, blue, yellow). Equilibration effects occurred within the first few ms (e.g., the red soma trace in panel C).
**Equivalent cylinder models**

Time constants of multi-exponential fits made to voltage responses to current steps were obtained from previous research (Chapter 5). This included five PY and four LP neurons. The two slowest non voltage-dependent time constants, $\tau_1$ and $\tau_2$, were used to calculate length constants for each neuron at different membrane potentials achieved by bias current injection. Input resistance varied across neurons, and within neurons, by membrane potential. To minimize activation of voltage-dependent currents, only measurements made between -50 mV to -80 mV were used. Neurons with $R_N < 4$ were excluded. Among LP neurons, $L$ ranged between 0.77 and 1.10, with mean $0.95 \pm 0.12$ (standard deviation); among PY neurons, $L$ ranged between was 0.92 and 1.50 with mean $1.17 \pm 0.13$ (*Figure 101*). As similar estimates were not available for other neuron types, the upper and lower bounds across both types, 0.75 and 1.5, were used as upper and lower estimate ranges for $L$ for equivalent cylinder models to represent pyloric neurons voltage clamped from -65 mV (rest) to -55 mV.

To produce equivalent cylinder models appropriate for voltage clamping under conditions of reduced input resistance, the electrotonic length was increased (see Materials and Methods – *Equivalent cylinder models*). In the traced neurons with flat capacitance adjustment method, and thus uniform membrane conductance (*Table 16*), the $G_m$ needed to achieve a nominal input resistance of 1 M$\Omega$ was approximately 15 times lower than the $G_m$ needed to achieve $R_{\text{nominal}} = 10$ M$\Omega$, i.e., $\bar{\rho}_{10} = 15$. For a hundred-fold decrease of input resistance to $R_{\text{nominal}} = 0.1$ M$\Omega$, $\bar{\rho}_{100} = 385$. Thus, lower and upper estimates of $L$ were multiplied by $\sqrt{15}$ and $\sqrt{385}$ to produce equivalent cylinder models appropriate for voltage clamping to -30 mV at $R_{\text{nominal}} = 1$ M$\Omega$, and to -5 mV at $R_{\text{nominal}} = 0.1$ M$\Omega$, respectively.
Five PY neurons (A,C; five different colors for the five neurons) and four LP neurons (B,D; four colors for the four neurons) neurons were stimulated with current pulses at multiple membrane potentials, and the voltage responses fit with a sum of exponentials. Excluding the slowest time constant due to an active voltage-dependent phenomena, the two slowest time constant coefficients were used to calculate an electrotonic length for an equivalent cylinder:

$$L = \frac{\pi}{\sqrt{\tau_1/\tau_2} - 1}$$

where $\tau_1$ is coefficient representing the passive membrane time constant, and $\tau_2$ is the first of the equalizing time constant coefficients (Rall, 1969). Electrotonic length was plotted against membrane potential (A,B) and input resistance (C,D).
Membrane voltages in the equivalent cylinder models were then calculated, and the range of membrane potentials across the middle 90%, 95%, and 99% of the cylinder were calculated. At $R_N = 10 \, \text{M}\Omega$, much of the cylinder was effectively clamped at $L = 0.75$, though, less so at $L = 1.5$. At lower input resistances, however, the cylinders were not effectively clamped, and especially at $R_N = 0.1 \, \text{M}\Omega$, much of the neuron remained near $V_{rest}$ (Figure 102). These results were similar to what was observed in traced pyloric neurons (Figure 99).

*Figure 102. Voltage versus axial electrotonic distance in equivalent cylinder models*

Equivalent cylinder models were made to simulate pyloric neurons voltage clamped to -55 mV at $R_N = 10 \, \text{M}\Omega$ (A), to -30 mV at $R_N = 1 \, \text{M}\Omega$ (B), and to -30 mV at $R_N = 1 \, \text{M}\Omega$ (C). Voltage was calculated for lower bound (red) and upper bound (blue) estimates of $L$ appropriate to the input resistance. Range of membrane potentials across 95%, 99%, and 99% (black bars) are shown.
Discussion

Current flow and membrane potential range

Conductance-based models of pyloric neurons, e.g., the Prinz model (Prinz et al., 2003), are often implemented in a single compartment. This is usually done for simplicity, but carries an underlying assumption that this is a sufficient approximation of a real neuron. This may indeed be correct in free-running conditions, in which the entire neuron slowly oscillates in synchrony, or when the neuron is near its resting potential and input resistance is correspondingly high. It would appear that even relatively small inhibitory synaptic currents (< 1 nA in the model neurons) are able to propagate throughout the entire neuron if input resistance is high, as it is during the hyperpolarizing phase of oscillatory behavior, the phase in which inhibitory input occurs. This likely supports the ability of these neurons to behave in a rhythmic fashion. Inhibitory drive in the depolarized phase of the oscillation, when input resistance is lower, is considerably less effective; this suggests that afferent inhibition from aberrant neurons which are out of phase with the pyloric rhythm might be effectively “quarantined”, contributing to the overall stability of the network.

Voltage clamping, however, is considerably less effective than synaptic current at driving the neuron. Especially at more depolarized clamp potentials, where input resistance is lower, large sections of the neuron remain at or near $V_{rest}$. This is due to spatial attenuation of current, not temporal filtering (Figure 100). This calls into question whether it is possible to effectively clamp the large currents that occur in depolarized regimes. The substantial range of potentials achieved within the neuron may encompass a very wide range of steady-state activation values ($m_{\infty}$) of voltage-dependent conductances (Figure 103). With the soma clamped in the -20 to -30 mV range, where one would expect to begin to see most of the voltage-dependent conductances begin to activate, much of the neuron would remain at or below -40 mV (Figure 99) at which
activation of most conductances is quite low. Even when conductances are activated and clamped, the potential at the soma is typically far from the local potential where the ion channels are located, potentially leading to erroneous measurements of voltage-dependent conductance parameters such as time constants, half-maximal activation voltage, and slope.

![Graph](image)

*Figure 103.* Steady-state activation gate values ($m_\infty^p$) of some Prinz model conductances. Activation gate state can vary considerably over relatively narrow membrane potential ranges.

These problems present a substantial challenge for modeling work which attempts to recover neuron parameters by optimization (Hobbs and Hooper, 2008). It may not be possible via voltage clamp to achieve the potentials required to activate some of the conductances to a degree which produces enough information to drive the optimization process. As such, current-clamp protocols, which allow depolarizing current to act synergistically with the neuron's own conductances to drive the dendritic arbor to more depolarized potentials, will likely be important tools in such work. Even assuming that sufficient current is obtained, the parameters are not likely to match the model if a single compartment is used, as the membrane potentials the ion channels experience do not correspond to the clamp potential. Multiple compartments would be
necessary to faithfully reproduce the range of membrane potentials that occur throughout the neuron.

Effect of adjustment method upon results

The choice of capacitance adjustment method affected results under some conditions. With both synaptic current propagation and action potential propagation, no significant differences were found between flat, linear, and terminal adjusted models. Propagation was however worse in extrapolated models. With simulated voltage clamp, the terminal adjusted models were significantly better clamped than the flat adjustment models, but otherwise no differences were identified among the adjusted models. This is consistent with prior research showing that nonuniform distribution of leak conductance with high distal leak supports current flow (London et al., 1999). Nonuniform leak distribution does occur in neurons (Johnston et al., 1996), so the terminal capacitance adjustment method may better approximate a real neuron than the flat capacitance adjustment method does. Again, clamping was significantly worse in the extrapolated models.

The terminal capacitance adjustment method and the extrapolated method were both designed to simulate untraced small terminal processes. The difference between the two is that the missing surface area in the former method is not included in the analysis, i.e., only the true (unadjusted) membrane surface area of the terminal compartments is measured. Thus the terminal capacitance adjustment method is useful to consider the spread of membrane potentials throughout the traced section of neuron, but it ignores the membrane potentials in the untraced neuron. This would be appropriate if these untraced processes are primarily passive in nature, and do not contain voltage-dependent conductances. The extrapolated method, which adds actual dendrite branches and includes them in measurement of membrane potentials, would be appropriate if the small terminal dendrites do contain active conductances.
Discrepancy between anatomical and electrophysiological capacitance measurements

Even when using proper current clamp measurement technique and excluding spurious slow exponential terms (Chapter 5), electrophysiological capacitance measurements are 2.5-5x higher than estimates made from anatomical traces. This discrepancy, though not as large as reported elsewhere (Golowasch et al., 2009), is nonetheless substantial. If true, it would mean that as much as 80% of the total membrane area was untraced. There may be many causes for the discrepancy. Capacitance measurements made using current clamp steps will overestimate capacitance by several-fold in the event of a large soma shunt current (Figure 3 of Golowasch et al., 2009). Since the capacitance measurements were performed using two-electrode impalement technique (Chapter 5), the presence of a soma shunt current is plausible, so this phenomenon alone may account for much of the discrepancy.

It is nonetheless likely that the traces are incomplete. Diffusion of dye molecules in small diameter dendrites is likely to be slow due to dendritic crowding (Biess et al., 2011). It is possible that the small diameter dendrites may have been incompletely penetrated by the fluorescent dye or that the intensity of fluorescence may not have been sufficient to trace these dendrites. While the tracing algorithm did obtain some diameter measurements below 1µm, most of the measured diameters were above 1 µm (Figure 91; note log scale). Thus, one source of the discrepancy may be that pyloric neurons contain many more small diameter dendrites than were actually traced. Interestingly, exponential fits to the charge curves of the compensated models often required multiple exponential terms, similar to real pyloric neurons (Chapter 5). Exponential fits to the unadjusted models did not, however, thus providing further evidence that the traces did not include the entire dendritic arbor. A second possibility is that the capacitance of the membrane may exceed 0.9 µF/cm². This could occur if the membrane specific capacitance is larger, or if the membrane itself is rough or crenulated and thus the membrane surface area in a
compartment exceeds that of a simple cylinder. The flat and linear adjustment methods attempt to simulate a greater membrane capacitance than expected, either throughout the neuron (flat) or primarily in more distal regions (linear).

Tracing with InteractiveTrace

The semi-automated tracing program, InteractiveTrace, is a promising alternative to existing neuron tracing packages such as NeuroLucida (Glaser and Glaser, 1990). Traditional methods produce anatomical models which are limited in detail and accuracy by the operator's experience, patience, and attention to detail. Many of the steps in InteractiveTrace are fully automated, utilizing statistical models when choices were required. Despite the relatively simple underlying logic, detailed models were obtained which accurately reflected the confocal images. Further automation in the tracing itself is limited in part by the computational resources, e.g., to precompute Fast Marching gradients; given the ever-decreasing price of high-performance computing resources, automated tracing may be feasible for future development.

Acknowledgements

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Disclosures

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CHAPTER 7: RECOMMENDATIONS FOR FUTURE RESEARCH

The obvious next step is to perform the equivalent of pathfinding in real neurons, by using a heuristic gradient descent method to try to discover parameter values for a model neuron that cause it to respond similarly to the real neuron. This research began because an early attempt to do so using white noise perturbation was not successful. Based upon this body of work, several recommendations can be made for future attempts.

First, perturbations other than white noise should be used. White noise was the worst performer in voltage clamp tests (Chapter 3) because white noise waveforms do not remain long at the depolarized or hyperpolarized potentials where voltage-dependent conductances are active. For voltage clamp, the composite optimized perturbation $cp1$ or an equivalent should be used. This includes a section of empirically optimized perturbation designed to elicit maximal conductance amplitudes, as well as other sections, e.g., clipped chirp, with good performance in separation and pathfinding tests. For current clamp, a composite perturbation containing the three best performing waveforms – pink noise, randomized chirp, and noisy chirp – is recommended.

Second, multiple distance metrics should be used. Different distance metrics can produce difference landscapes with intersecting, even orthogonal valleys. Spike timing metrics such as modified Fiducial-point distance (Ambros-Ingerson et al., 2008) should also be considered if sodium spikes are present (see below). Waveform distance in particular is highly sensitive to frequency differences with long sample times, though this is only applicable to rhythmic activity produced in current clamp. This is a liability in gradient descent, because it means that the target is likely to be in a steep valley with high ridges surrounding it. Thus, for spontaneous rhythmic activity, sample times should be shorter, or waveform distance should be avoided or modified somehow to prevent this problem.
Third, the fast sodium conductance should not be blocked. This research, and much of
the research in our lab, was conducted using picrotoxin (PTX) and tetrodotoxin (TTX) to
chemically isolate pyloric neurons. Instead, PTX can be used with low concentrations of TEA to
isolate most neurons and leave sodium spikes intact. This has several advantages. Penetration of
voltage clamp current into the dendritic arbor is poor (Chapter 6), whereas action potentials may
provide additional depolarizing current to reach deeper into the dendritic arbor during current
clamp. With sodium spikes intact, neurons retain rhythmic output, and this may provide
additional information for fitting. Finally, the presence of fast sodium spikes makes it possible to
use additional distance metrics based on spike timing, such as Fiducial-point distance.

Fourth, the neuron model should be reevaluated. The Prinz model does not adequately fit
the behavior of real pyloric neurons (Chapter 4), in part due to incorrect dynamics of its $I_{H}$. At
the very least, the equations for this conductance should be corrected with experimental data
taken from pyloric neurons. A persistent sodium current, $I_{NaP}$, should also be considered for
inclusion. And the cause of the slow relaxation to rest potential that occurs during current clamp
depolarizations in some neurons (Figure 58) should be investigated, as this may indicate a novel
current or suggest that the dynamics of chloride re-equilibration are fast enough to contribute to
neuronal response.

Finally, the corrected neuron model should be implemented in multiple compartments.
Especially at more depolarized potentials, when input resistance is low, pyloric neurons are very
non-isopotential. At least three or four compartments (e.g., soma, proximal dendrites, distal
dendrites, and axon) may be needed to adequately represent the distribution of membrane
potentials throughout the neuron.


King DG (1976) Organization of crustacean neuropil. II. Distribution of synaptic contacts on identified motor neurons in lobster stomatogastric ganglion. J Neurocytol 5:239-266.


Rall W, Rinzel J. (1973) Branch input resistance and steady attenuation for input to one branch of a dendritic neuron model. pp 648-688.


Ward JH (1963) Hierarchical grouping to optimize an objective function. pp 236-244.
APPENDIX 1: SOFTWARE IMPLEMENTATION DETAILS

Overview of software suite

The bulk of the computational modeling and other tasks were performed utilizing custom software libraries written in C, with some additional software in C++ and Perl. The base library (libhooperlab) provided common structures and functions for model parameters, local optimization, parallel computing infrastructure, numerical integration, and analysis. This base library included implementation of several distance metrics such as waveform distance (D_w) and phase plane distance (D_P), as well as frequency detection. Additional libraries provided implementation of specific models, including the Prinz model (Prinz et al., 2003), Prinz+I_{NaP} model, single and multiple compartment passive models, and others. Source code for these libraries and associated applications will be made available for download at http://crab-lab.zool.ohiou.edu/bill/modeling/

Many applications were developed; most of these were straightforward. A few require further description than was offered in the chapters of the dissertation. These are described in detail below.

Vector Balloon

The vballoon (vector balloon) program was used to provide seed data for high resolution flood-fill explorations of parameter space to determine the extent of the basin of equivalence for a reference model neuron. When the program was written, prior research (Achard and De Schutter E., 2006) suggested that these regions may be only loosely connected. Also, while it was known that the Prinz model was more sensitive to some parameters than others, it was not known whether the sensitivity might vary across parameter space, or what appropriate scale factors were to normalize this differing sensitivity. Thus, an algorithm was designed which would be able to explore widespread of parameter space connected only by thin regions, and could do so without a
priori knowledge of the sensitivity of response to each parameter. It functioned by repeatedly choosing random vectors, starting from an already explored region of parameter space, moving along that vector in discrete steps of increasing size, until leaving the basin of equivalence. The start and end points of the new vector were then added to the pool of explored points, and the process continued. Three periodically changing bias parameters were used to affect how new vectors were chosen, under the assumption that what provides good performance in one region of parameter space might not do so in another.

The vector balloon program maintained a set of “seeds”, \( S \). Each member of this set was a pair of parameter space points describing the start and end of a line segment. The initial member of this set was the zero-length line segment \((p_{\text{ref}}, p_{\text{ref}})\), i.e., the point characterizing the reference model neuron whose basin of equivalence was being explored. A member \( s = (a, b) \) was chosen from \( S \). Then, a point \( p_{\text{init}} \) was chosen between \( a \) and \( b \). If \( p_{\text{init}} \) was not within the basin of equivalence, which could for example happen when \( a \) and \( b \) were in a highly curved region, a new point was chosen. A random vector \( v \) was chosen in parameter space, the step size \( x \) was initialized to 1, and the test point \( p \) was set to \( p_{\text{init}} \). Then, the program calculated \( D_W(p + x \cdot v, p_{\text{ref}}) \). So long as this was \( \leq \varepsilon \), the parameter values \((p + x \cdot v)\) were written to the output file, \( p \) was incremented to \( p + x \cdot v \), and \( x \) was increased by 10%. Once the boundary of the basin of equivalence was exceeded, \( x \) was repeatedly decreased by 10%, until it was either below 1, or \( D_W(p + x \cdot v, p_{\text{ref}}) \) was back inside the basin. The initial random point \( p_{\text{init}} \) and the most distant \( p \) within the basin, were added to \( S \) as a new seed, \((p_{\text{init}}, p)\). Then the process began again, with a new seed and vector.

Choosing a seed from the set \( S \) was done as follows. For each \((a, b)\) in \( S \), a distance score \( d \) was assigned, \( d = \max(|a - p_{\text{ref}}|, |b - p_{\text{ref}}|) + |a - b| \cdot k_1 \), where \( k_1 \) was one of the three bias parameters. The first part of the distance score, \( \max(|a - p_{\text{ref}}|, |b - p_{\text{ref}}|) \), measured the distance from the farther
end of the \((a, b)\) line segment to the original seed point (the reference \(p_{\text{ref}}\)). The second part, \(|a-b|k_1\), biased the score in favor of shorter or longer \((a, b)\) line segments. The bias parameter calculations are described below. The entries in S were then sorted in order of decreasing distance score. An index into the sorted table was chosen randomly, with Gaussian distribution with \(\mu = 0\) and \(\sigma = 1/k_2\), another bias parameter, used to control whether the random selection had a strong or weak preference for high-scoring entries. The pair at this index was then used; when the index was less than zero, the first entry in the table (i.e., smallest distance) was selected.

The point \(p_{\text{init}}\) between \(a\) and \(b\) was selected by first arranging \(a\) and \(b\) in order of distance from \(p_{\text{ref}}\) (i.e., so that \(b\) was farther). Then a random number \(r\) was selected from a Gaussian distribution with \(\mu = 0\) and \(\sigma = 1/(k_3+0.01)\); this bias parameter affected how strongly the program preferred the distant end of the line segment. This \(r\) was then bounded within \([0,1]\). When \(\sigma > 5\), a flat distribution \([0,1]\) was used instead. \(p_{\text{init}}\) was then set to \(b + r(a-b)\).

The three bias parameters were constantly changed over time:

\[
\begin{align*}
\theta &= 2\pi n/5000 \\
k_1 &= 0.1 \cdot \cos(\theta/3) \\
k_2 &= \cos(\theta/2) + 1 \\
k_3 &= 0.7 \cdot (\cos(\theta) + 1)
\end{align*}
\]

where \(n\) was the number of program iterations. This allowed the bias parameters to change in such a way that they would cycle between maxima and minima with different periods, achieving many possible combinations of values. This allowed the program to explore in many regions of parameter space, some of which might require different optimal conditions than others. Exploration with the use of these bias parameters was faster than without, especially in highly curved portions of the basin of equivalence.
The output from the vector balloon program, comprising points discovered within the basin of equivalence, was then utilized by the flood fill program. This latter program aligned the vector balloon output points to a grid and then performed a standard flood fill, as described in Chapter 2. These programs were used to explore locally connected regions of parameter space. To explore additional regions which were not locally connected, an alternative method is suggested, using the raindrop program (Chapter 3) to find many local minima within the basin of equivalence throughout parameter space, and then initiating the vector balloon and flood fill from those seed points.
Evolution Strategy

The evolution strategy used in this research was an extension of a standard evolutionary algorithm (Yao X et al., 1999; Fogel, 1994). The extensions were implemented to increase its performance at the task of evolving waveform parameters for the conductance optimized perturbations (Chapter 3), and as such, the tuning parameters used are probably specific to that task. These tuning parameters were determined through trial and error. The tuning and other parameters of the evolution strategy are reported in Table 17; these are specified in boldface below. This section expands upon the conceptual description given in Chapter 3 by explicitly describing the different stages of the algorithm.

Definitions and notation

In the evolution strategy, an individual is a data structure which contains several different components. Each individual is assigned a number, designated $i$. These components include:

1. The individual’s genotype, in this case, the set of $n_{\text{param}}$ real-valued waveform parameters to be optimized.
2. The mutation sizes, one corresponding to each waveform parameter. These are used as standard deviations for the mutation step and thus govern the degree of mutation.
3. The colony number, designated $\zeta$, which specifies to which colony the individual belongs.
4. The drift counter, designated $\beta$, specifying how many more generations remain before the individual is placed into the main colony (i.e., colony #1).
5. The fitness value, $\phi$, a real number giving the fitness score associated with the waveform parameters. In this implementation, lower fitness scores were more desirable. The fitness function is described below. The fitness value of a newly constructed individual is undefined.
The *population* is the collection of individuals participating in evolution, and includes a fixed number of individuals designated *parents*, and an equal number of individuals designated *children*.

Random numbers are used throughout the evolution strategy. These are referred to as:

- $R_{flat}(a,b)$ is a random number taken from a flat distribution ranging from $a$ to $b$
- $R_{norm}(\sigma)$ is a random number taken from a Gaussian distribution with standard deviation $\sigma$
- $R_{norm}(\mu,\sigma)$ from Gaussian distribution with mean $\mu$ and standard deviation $\sigma$
- $R_{cauchy}(s)$ from a Cauchy distribution of size $s$

*Genotype*

The genotype of an individual was the set of waveform parameters corresponding to a waveform comprising connected ramp segments (*Figure 25*). Each pair of waveform parameters represented the amplitude at the end of a ramp segment and its duration. The amplitudes at the beginning and end of the first ramp were identical, allowing the algorithm to begin the waveform with a lead-in holding potential. Amplitudes were constrained between -5 mV and -100 mV, and durations between 0.05 ms and 50 ms. Each ramp was further limited to a maximum $dV/dt$ of 10 mV/ms, to prevent damaging currents or ringing if used in voltage clamping real neurons.

*Fitness function*

The fitness function was hand tuned to provide good performance with the conductances of the Prinz+I\textsubscript{NaP} model. It functioned as follows. $I_j$ refers to the $j$th of the 9 currents of the model ($I_{CaT}$, $I_{CaS}$, $I_{KCa}$, $I_A$, $I_{Kd}$, $I_{H}$, $I_{Na}$, $I_{NaP}$, and $I_{cap}$, the latter being the capacitance current):

$$
\phi_j = \int_0^d 9 \cdot |I_j(t)|^{1.2} - \sum_{k=1,k\neq j}^9 w_j(t) u_{jk}(t) |I_k(t)|^{1.5} \ dt + P
$$
This set the fitness score for current \( j \) according to the amplitude of that current \( (I_j) \), and the amplitude of the remaining currents \( (I_k) \) modified by two adjustment factors \( (w_j, u_{jk}) \) and a penalty score for high \( dV/dt \) \( (P) \). The adjustment factors were calculated as follows:

\[
\begin{align*}
    w_j(t) &= 0.1 + I_j(t)/2000, \text{constrained in } [0.2,1.0] \\
    u_{jk}(t) &= \begin{cases}
        10 & \text{where } I_j \text{ is } I_{KCa} \text{ and } I_k \text{ is } I_{Kd} \\
        3 & \text{where } I_j \text{ is } I_{Kd} \text{ and } I_k \text{ is } I_{KCa} \\
       1 & \text{otherwise}
    \end{cases}
\end{align*}
\]

The first increased the penalty assigned to unwanted currents in proportion to the amount of the target current \( (I_j) \) being produced. The second prevented local minima from arising when trying to evolve currents for \( I_{KCa} \) or \( I_{Kd} \). The \( dV/dt \) penalty was calculated as:

\[
P = 10 \cdot \sum_{i=1}^{n_{\text{param}}-1} \frac{2|V_i - V'_i|}{n}
\]

\[
V'_i = V_{i-1} + \frac{V_{i+1} - V_{i-1}}{t_{i+1} - t_{i-1}} \cdot (t_i - t_{i-1})
\]

Where \( V_i \) and \( t_i \) are the voltage and time of the \( i \)th sample of the waveform being evolved.

Initialization

Each individual \( i \) was initialized as follows. The first two steps were standard.

- Genotype parameters, \( x_{ij} \) where \( j = 1..n_{\text{param}} \), were set to \( R_{\text{flat}}(imin_j,imax_j) \), where \( imin_j \) and \( imax_j \) are the initial minimum and maximum values for the \( j \)th parameter (for amplitudes, -80 mV to -20 mV; for ramp durations, 5 ms to 20 ms)
- Mutation sizes: \( \eta_{ij} = 0.02 (imax_j - imin_j) \)
- Colony number: \( \zeta_i = i \mod n_{\text{colonies}} \)
- Drift count: \( \beta_i = \text{floor}(R_{\text{norm}}(\mu_{\text{colonies}}, \sigma_{\text{colonies}})) \)
Fitness scoring

Each generation started with fitness scoring in any individuals with undefined fitness scores. In the first generation this included all parents; in subsequent generations, all children.

Selection

Individuals competed only within a colony. Competition rules varied by colony. If \((\frac{\zeta}{2}) \) % 2 = 1 (colonies 2, 3, 6, 7, …), the colony was “elitist”, otherwise it was “non-elitist”. In elitist colonies, each individual (parents and children) competed against every other individual in the colony. In non-elitist colonies, each individual competed against \(n_{\text{compete}}\) randomly chosen other individuals. In each round of competition, a win was assigned to the individual having the better (lower) fitness score. The members of the colony were sorted according to number of wins, with lower numbered individuals having more wins, and the parents in the colony replaced by the winners in the round of selection.

Mutation

Mutation was standard. During mutation all children were replaced, with each parent spawning one child in the same colony with an uninitialized fitness value. The mutation occurred as follows:

\[
\eta'_{ij} = \eta_{ij} \cdot e^{\tau' r_i + \tau R_{\text{norm}}(0,1)}
\]
\[
x'_{ij} = x_{ij} + \eta'_{ij} \cdot R_{\text{cauchy}}(1)
\]

with \(r_i\) being a random number drawn once per individual from \(R_{\text{norm}}(1)\). The colony number and drift time, \(\zeta_i\) and \(\beta_n\), remained the same. The two \(\tau\) values are

\[
\tau = \frac{1}{\sqrt{2 \cdot n_{\text{param}}}}
\]
\[
\tau' = \frac{1}{\sqrt{2 \cdot \sqrt{n_{\text{param}}}}}
\]
Recombination

For each child individual $i$ in the colony, if a random number $R_{\text{flat}}(0, 1) < p_{\text{recom}}$, then the individual was chosen for recombination. If the colony number was even (2, 4, 6, ...), then the individual’s genotype was replaced entirely by the combined genotype of two randomly chosen parents. The number of crossover points was first chosen:

$$n_x = R_{\text{norm}}(\mu_x, \sigma_x), \text{constrained } 1 \leq n_x \leq \frac{n_{\text{param}}}{10}$$

i.e., up to 10% of the total number of parameters. The specific crossover positions $\chi_j (j = 1..n_x)$ were selected:

$$\chi_j = R_{\text{flat}}(0, n_{\text{param}}), \text{for } j = 1..n_x$$

and two parents were chosen to provide the replacement genes:

$$i_{\text{mother}} = R_{\text{cauchy}}(\sigma_{\text{mother}}), \text{constrained } 0 \leq i_{\text{mother}} < n_{\text{pop}}$$

$$i_{\text{father}} = R_{\text{cauchy}}(\sigma_{\text{father}}), \text{constrained } 0 \leq i_{\text{father}} < n_{\text{pop}}$$

Then, the child’s genotype was replaced by alternating between the mother and father’s genotype:

$$\text{for } j = 0..n_{\text{param}} - 1,$$

$$k = 1 - k \text{ if } j \in \chi$$

$$\text{child } x_{ij} = \begin{cases} \text{parent } x_{ij_{\text{father}}} \text{ where } k = 0 \\ \text{parent } x_{ij_{\text{mother}}} \text{ where } k = 1 \end{cases}$$

This iterated through the waveform parameters, alternating between the mother’s waveform parameter or the father’s waveform parameter at crossover points.

If the colony number was odd, then a random second child was chosen from $R_{\text{flat}}(0, n_{\text{pop}})$, and the two children exchanged genetic material through an identical crossover process.
Genetic drift

In the final step in a generation, each individual’s drift counter was decremented, and if it reached 0, the individual was merged back into the main colony. Then, the entire population was assessed for stagnation. Either of two criteria could be met to consider a population stagnant: first, the fittest 10% of individuals, or at least 20% of all individuals, had not changed in the past generation; second, the top-ranked individual had not changed for 40 generations and at least 25% of individuals had not changed in the past generation. The first time a population was considered stagnant, the “stall generation number”, \( t_{\text{stall}} \), was set to the current generation number; this was used later to determine the ideal number of generations a new colony should remain isolated.

Once a population was stalled, every fifth stalled generation, a random action was taken:

- 50% of the time, nothing occurred
- 10% of the time, the recombination parameters \( P_{\text{recom}} \), \( \mu_x, \sigma_x, \sigma_{\text{mother}}, \sigma_{\text{father}} \), and the competition size \( n_{\text{compete}} \) were replaced with random numbers from the distributions shown in Table 17. Note that these are global parameters applying to all individuals.
- 10% of the time, a randomly selected subset of individuals (chosen with 10% probability in the base colony, 2% probability otherwise) had their mutation parameters \( \eta_{ij} \) reset to initial values. Mutation sizes typically decreased throughout the course of evolution, causing offspring to be increasingly similar to parents. Resetting mutation sizes thus gave these individuals greater mutability.
- 30% of the time, genetic drift occurred by creation of a new colony, unless a new colony had recently been created (see below). The new colony’s ID number, \( \zeta \), was set by incrementing the number of the last created colony, modulo \( n_{\text{colonies}} \). Then, all parents in
the base colony, except the parent with highest fitness, was selected with 20% probability, and those selected were assigned to the new colony by setting their $\zeta_i$ to the new colony number. Every individual placed into the new colony had their drift time set as $\beta_i = t + R_{\text{norm}}(1.5 \ t_{\text{stall}} \ (1.5 \ t_{\text{stall}})/10)$, and at least $t+40$, $t$ being the current generation number. All individuals chosen to populate the new colony had their mutation sizes reset to individual conditions. 90% of the time, their genotypes were also reset to initial conditions. To prevent colonies from spawning too frequently, a delay was implemented; the minimum generation for a new colony, $t_{\text{next}}$, was set to $t + 1.5 \ t_{\text{stall}}$, or at least $t+50$.

The choice of probabilities and of the tuning constants was somewhat arbitrary. Once the performance of the evolution strategy was considered adequate, no attempt was made to further adjust these parameters. While the overall tuning process greatly increased the performance of the algorithm both in speed and in local minima avoidance, the degree to which any particular tuning parameter’s value contributed to the performance improvement is not known.
<table>
<thead>
<tr>
<th>Param</th>
<th>Initial value</th>
<th>Mutator</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n_{\text{param}}$</td>
<td>128</td>
<td></td>
<td>No. of waveform parameters</td>
</tr>
<tr>
<td>$n_{\text{pop}}$</td>
<td>2000</td>
<td></td>
<td>Size of population</td>
</tr>
<tr>
<td>$\text{imin}_j$</td>
<td>5 ms, -80 mV, -8 nA</td>
<td></td>
<td>Initial min $dt$, voltage, or current</td>
</tr>
<tr>
<td>$\text{imax}_j$</td>
<td>10 ms, -20 mV, 8 nA</td>
<td></td>
<td>Initial max $dt$, voltage or current</td>
</tr>
<tr>
<td>$\text{min}_j$</td>
<td>0.05 ms, -100 mV, -20 nA</td>
<td></td>
<td>Min $dt$, voltage, or current</td>
</tr>
<tr>
<td>$\text{max}_j$</td>
<td>50 ms, -5 mV, 20 nA</td>
<td></td>
<td>Max $dt$, voltage, or current</td>
</tr>
<tr>
<td>$n_{\text{colonies}}$</td>
<td>16</td>
<td></td>
<td>Max colonies</td>
</tr>
<tr>
<td>$\mu_{\text{colonies}}$</td>
<td>400</td>
<td></td>
<td>Drift count randomizer</td>
</tr>
<tr>
<td>$\sigma_{\text{colonies}}$</td>
<td>15</td>
<td></td>
<td>Drift count randomizer</td>
</tr>
<tr>
<td>$n_{\text{compete}}$</td>
<td>2000</td>
<td>$2000 e^{R_{\text{norm}(1)}}$</td>
<td>Competition size</td>
</tr>
<tr>
<td>$p_{\text{recom}}$</td>
<td>0.1</td>
<td>$R_{\text{norm}}(0.25,0.2)$ in [0,0.8]</td>
<td>Prob. of recombination</td>
</tr>
<tr>
<td>$\mu_x$</td>
<td>3</td>
<td>$R_{\text{norm}}(3,2)$ in [1,10]</td>
<td>Mean crossover count</td>
</tr>
<tr>
<td>$\sigma_x$</td>
<td>1</td>
<td>$R_{\text{norm}}(2,2)$ in [0.1,10]</td>
<td>Crossover count std dev</td>
</tr>
<tr>
<td>$\sigma_{\text{mother}}$</td>
<td>10</td>
<td>$R_{\text{norm}}(20,5)$ in [4,200]</td>
<td>Mother selection std dev</td>
</tr>
<tr>
<td>$\sigma_{\text{father}}$</td>
<td>100</td>
<td>$R_{\text{norm}}(80,10)$ in [4,200]</td>
<td>Father selection std dev</td>
</tr>
</tbody>
</table>

In [a,b] means that the random number was bounded within [a,b] by reselection if out of bounds. Waveform parameters alternated between duration values and voltage (voltage clamp) or current (current clamp) values.